

Ecology and Biological Control of

Verticillium dahliae

Promotor: prof. dr M.J. Jeger
 hoogleraar in de ecologische fytopathologie

Co-promotor: dr A.J. Termorshuizen
 universitair docent bij de leerstoelgroep biologische bedrijfssystemen

Propositions

1. With *Arabidopsis thaliana*, microsclerotia of *Verticillium dahliae* can be quantified down to 1 microsclerotium g⁻¹ soil. (*This thesis*).
2. Rather than direct detection methods such as plating techniques, bioassays integrate information on multiple soil factors leading to infection by *Verticillium dahliae*. (*This thesis*).
3. The dynamics of formation of microsclerotia of *Verticillium dahliae* in senescent host tissue is the result of a complex interaction between weather, host growth, number of root infections, and host senescence rate. (*This thesis*).
4. In quantifying microsclerotia of *Verticillium dahliae* in soil it should be taken into account that the amount of recoverable microsclerotia may change in time. (*This thesis*).
5. Consistency of biological control can be improved by applying compatible combinations of antagonists. (*This thesis*).
6. Biological control of plant pathogens will improve the quality of the environment.
7. Absence of above-ground symptoms of *Verticillium dahliae* does not exclude the presence of a pathogenic interaction below-ground.
8. Educating farmers in farmer's schools improves management of plant diseases and therefore leads to more sustainable agriculture.
9. Wisdom is knowing when to speak your mind and when to mind your speech. When we are filled with pride, we leave no room for wisdom (*Our Daily Bread, RBC Ministries, Grand Rapids, Michigan, 1999*).
10. We may not complain about so many thorns on roses but we should be thankful for the roses among thorns.

11. Proper education is decisive for the quality of our future generations.

12. Understanding, paying attention, hospitality, and self control are the basis for building up good relationships among people having different cultural, religious, and social backgrounds.

Propositions attached to the thesis: **Ecology and Biological Control of *Verticillium dahliae*** by L. Soesanto, defended on 29 of March 2000.

L. Soesanto

**Ecology and Biological Control of
*Verticillium dahliae***

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
dr C.M. Karssen,
in het openbaar te verdedigen
op woensdag 29 maart 2000
des namiddags te 16.00 uur in de Aula
van Wageningen Universiteit

1501 970665

Bibliographic data

Soesanto, L., 2000

Ecology and biological control of *Verticillium dahliae*

PhD Thesis Wageningen University, Wageningen, the Netherlands

With references - With summary in English, Dutch, and Indonesian

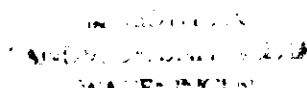
ISBN 90-5808-192-3

Cover design: P.J. Kostense and L. Soesanto

The studies described in this thesis were performed at the Laboratory of Phytopathology and Biological Farming Systems, the Department of Crop Science, Wageningen University, Marijkeweg 22, 6709 PG Wageningen, the Netherlands.

The research was fully financed by the Minister of Education and Culture, the Republic of Indonesia, through the Higher Education Development Project (HEP II) - ADB Loan No. 1235-INO.

Printed by Ponsen & Looijen B.V.



Dedicated to
my mother and my late father
who passed away on 17th of December 1998,
to my wife and my brothers and sister and their family:
Jones, Denny, Eli, Yenny, and Agus

ABSTRACT

Soesanto, L., 2000. Ecology and biological control of *Verticillium dahliae*. PhD Thesis, Wageningen University, Wageningen, the Netherlands.

The dynamics of *Verticillium dahliae*, the causal agent of wilt disease in many crops including potato, cotton, and olive, were investigated. Its biological control with *Talaromyces flavus* with or without additional *Pseudomonas fluorescens* was attempted. *Arabidopsis thaliana* was selected as a bioassay plant for studying aspects of ecology and biological control of the pathogen because of its short life cycle and high sensitivity to the pathogen. The optimal temperature for production of microsclerotia, the survival structures of the pathogen, both *in vitro* and on *A. thaliana* was about 20°C. Microsclerotia incorporated in soil were exposed to a range of conditions of temperature and moisture and quantified on several sampling occasions. One day after incorporation, densities were low, and were even lower over the following 1-6 months, but subsequently densities increased. These changes were ascribed to change in the level of soil mycostasis rather than death and new formation of microsclerotia. After application of *T. flavus* to fresh organic debris containing microsclerotia followed by a 3-week incubation aboveground at 15 or 25°C the population density of *T. flavus* increased in soil, especially at 25°C. *T. flavus* significantly reduced the density of microsclerotia in soil, especially at 25°C, and delayed the development of senescence of *A. thaliana* at 15 and 25°C. It is concluded that above-ground application of *T. flavus* may lead to more consistent effects. The effect of *P. fluorescens* strain P60, originally isolated from a take-all decline field continuously grown to wheat, on *V. dahliae* was also studied. Strain P60, and two other isolates of *P. fluorescens*, inhibited the *in vitro* mycelial growth of 20 isolates of *V. dahliae*, reduced formation of microsclerotia both *in vitro* and on *A. thaliana* and they retarded senescence of *A. thaliana* to a rate like that of uninoculated plants.

Key words: *Arabidopsis thaliana*, biological control, bioassay, detection, eggplant, microsclerotia, *Pseudomonas fluorescens*, recovery, relative humidity, *Solanum melongena*, *Talaromyces flavus*, temperature, *Verticillium dahliae*.

CONTENTS

Preface	11
Chapter 1. General introduction	13
Chapter 2. <i>Arabidopsis thaliana</i> as a bioassay plant for <i>Verticillium dahliae</i>	25
Chapter 3. Effect of temperature on formation of microsclerotia of <i>Verticillium dahliae</i>	37
Chapter 4. Recovery of microsclerotia of <i>Verticillium dahliae</i> from soil as subjected to various treatments	49
Chapter 5. Above-ground application of <i>Talaromyces flavus</i> to control <i>Verticillium dahliae</i>	61
Chapter 6. Control of <i>Verticillium dahliae</i> in <i>Arabidopsis thaliana</i> and eggplant (<i>Solanum melongena</i>) by combination of <i>Pseudomonas fluorescens</i> and <i>Talaromyces flavus</i>	73
Chapter 7. General discussion	89
References	95
Summary	107
Samenvatting	111
Ringkasan	115
Author's curriculum vitae	120

PREFACE

This thesis is the result of four years of investigating the ecology of the plant pathogen *Verticillium dahliae* carried out at the Laboratory of Phytopathology, section Ecological Phytopathology, Wageningen University, the Netherlands.

First of all I deeply would like to thank God for His mercy to follow a PhD program, for His guidance and strength during this hard time, and for His blessing to finish the program and defend my thesis. It's true that "I can do everything through Him who gives me strength" (Philippians 4:13).

This research was initiated in September 1995 under the supervision of Dr A.J. Termorshuizen, recently moved to the Group of Biological Farming Systems, Department of Plant Science, Wageningen University. I am very grateful for his persistent guidance, help, understanding, and support from the beginning up to the end of the research. Aad, thank you very much for everything you have done to encourage me. I would like to thank Prof. Dr M.J. Jeger for his efforts to attain the promotion. I acknowledge my gratitude to Dr J.M. Raaijmakers for his encouragement, supervision, motivation, and reading the manuscript.

The project was granted by the Ministry of Education and Culture, the Republic of Indonesia, through the Higher Education Development Project (HEP II) - ADB Loan No. 1235-INO, to whom I am sincerely grateful for the financial support. I also extend my sincere gratitude to Nuffic both in Jakarta, Indonesia, and in The Hague, the Netherlands, for facilitating my stay in the Netherlands.

I deeply would like to thank the Rector of the University of Jenderal Soedirman (UNSOED) and the Dean of the Faculty of Agriculture, UNSOED, Purwokerto, Indonesia, for their permission and support to improve my appreciation of science at Wageningen University. I am determined to distribute my acquired skills to UNSOED. My thanks also go to my colleagues at the Department of Plant Pests and Diseases, the Faculty of Agriculture, UNSOED. The Dean's Office for International Students, WAU, arranged and managed my accommodation, extension, and travel. I am very grateful for their service.

I would like to thank Prof. Dr W. Gams (Centraalbureau voor Schimmelcultures, Baarn), Dr W.J. Blok (Laboratory of Phytopathology), Dr J.A. Hiemstra (CPRO, Wageningen), and Dr M.P.M. Nagtzaam (LNV, The Hague) for useful comments on earlier versions of the manuscript.

I further thank all the members of the Laboratory of Phytopathology with special thanks to the section of Ecological Phytopathology: Dine Volker, Wout Hoogkamer, Drs Gerrit Bollen, Dr Gerrie Tuitert, Dr Wim Blok, Dr Mario Nagtzaam, Jan-Kees Goud, Trudie Coenen, Dr Gitte Schober, Eelco Gilijamse, Herman Frinking, Jorge de Souza, Gerard van Leeuwen, Angelique

Lamour, and Corrie Geerds for their help and nice co-operation. For some of the fellow students, Hans Meerman, Pella Brinkman, Dione Bouchout, Gerben Messelink, Dereje Assefa Abera, and other students for their kind co-operation during working in a pleasant atmosphere, I extend my thanks. Special thanks go to Willem Twijssel and his family for their kind support and companionship. I give my thanks to Drs Gerrit Gort (section of Mathematics) for his statistical advices. I also thank the management of the greenhouse and climate chamber facilities, Unifarm, for assistance during my experiments. Wim, Bertus, Pieter, Rick, Casper, and Rene, many thanks! I appreciate the Library of FYTO (Heleen, Ans) and Duotone for their service. I really thank you.

I wish to thank the Indonesian Student Association of Wageningen and the whole family of Indonesian communities, with special thanks go to Mrs. Widjorini Eveleens and her family, the International Student Chaplaincy, the International Christian Fellowship, and my foreign friends, for their hospitality, spiritual support, and kind co-operation during studying in the Netherlands. Also many thanks go to those I did not mention here for their ideas and support.

Finally, no list of acknowledgements would be completed without an acknowledgement of very special gratitude to my mother and my late father who invested in education but did not live long enough to see its result, to Mrs. Sri Rahayu and Mrs. Imam Soetoro with their family, to my dear wife, and my brothers and sister: Jones, Denny, Eli, Yenny, and Agus, with their family. Very special thanks go to you all for your support, encouragement, and understanding, during my study in the Netherlands. *Akhirnya, tiada senerai ucapan terima kasih yang lengkap tanpa ungkapan rasa terima kasih yang sangat tulus dan hormat kepada ibunda dan almarhum ayahnda yang telah membekali kami dengan pendidikan tetapi tidak hidup cukup lama untuk menikmati hasilnya, kepada ibunda Sri Rahayu dan ibunda Imam Soetoro beserta keluarga, kepada istriku tercinta, dan adik-adikku: Jones, Denny, Eli, Yenny, dan Agus beserta keluarga mereka masing-masing, atas dorongan, semangat, dan pengertian yang telah diberikan khususnya selama masa-masa sukar.*

Wageningen, January 2000

CHAPTER 1

GENERAL INTRODUCTION

Verticillium dahliae Kleb. is a soilborne pathogen that causes wilt in a wide range of hosts (Schnathorst, 1981). It affects many crops, the most economically significant being cotton, potato, and olive. In addition many other crops can be affected seriously, including vegetables (artichoke, eggplant, cauliflower, pepper), fruits (strawberry, grapevine), fruit trees (avocado, cocoa), stone fruit, fibre and oil seed crops (apricot, kenaf (*Hibiscus cannabinus*), linseed, sunflower), ornamentals (chrysanthemum, dahlia, rose), and shade trees (maple, ash). Some reports on severe outbreaks of *V. dahliae* are listed in Table 1.1.

Table 1.1. Reports of severe yield losses caused by *Verticillium dahliae*.

Major hosts	Location	Yield loss	References
Almond (<i>Prunus dulcis</i>)	Italy	- ¹	Luisi and Sicoli, 1993; Luisi <i>et al.</i> , 1994
Artichoke (<i>Cynara scolymus</i>)	Chile	22-70%	Fernandez and Tobar, 1989
"	Italy	-	Cirulli <i>et al.</i> , 1994
Ash (<i>Fraxinus</i> spp.)	USA	20-30%	Heffer and Regan, 1996
Avocado (<i>Persea</i> spp.)	Chile	-	Latorre and Allende, 1983
Cauliflower (<i>Brassica oleracea</i> var. <i>botrytis</i>)	USA	95%	Koike <i>et al.</i> , 1994
Cocoa (<i>Theobroma cacao</i>)	Brazil	85%	Resende <i>et al.</i> , 1994
Cotton (<i>Gossypium hirsutum</i>)	China	-	Shi <i>et al.</i> , 1998
"	Spain	-	Bejarano-Alcázar <i>et al.</i> , 1996
"	USA	100%	Friebertshausen and DeVay, 1982
Eggplant (<i>Solanum melongena</i>)	Japan	-	Hashimoto, 1989
Horseradish (<i>Armoracia rusticana</i>)	USA	75%	Eastburn and Chang, 1994
Linseed (<i>Linum usitatissimum</i>)	UK	>98%	Fitt <i>et al.</i> , 1991; 1992
Olive (<i>Olea europaea</i>)	Greece	-	Thanassouloupoulos 1993
"	Spain	25%	Blanco-López <i>et al.</i> , 1984
Paprika (<i>Capsicum annuum</i>)	Israel	22%	Tsrar <i>et al.</i> , 1998
Potato (<i>Solanum tuberosum</i>)	Canada	-	Platt <i>et al.</i> , 1995
"	USA	50%	Rowe <i>et al.</i> , 1987
"	Israel	20-50%	Susnoschi <i>et al.</i> , 1974
Protea (<i>Leucospermum cordifolium</i>)	USA	-	Koike <i>et al.</i> , 1991
Pyramid tree (<i>Lagunaria patersonii</i>)	Italy	-	Polizzi, 1996
Sunflower (<i>Helianthus annuus</i>)	UK	25-90%	Church and McCartney, 1995
Winter rape (<i>Brassica napus</i>)	Germany	44%	Daebeler <i>et al.</i> , 1988

¹ Precise yield loss not indicated.

An extensive host list has been presented by Pegg (1974). Monocotyledonous plants are generally considered non-hosts, with the notable exception of barley (Mathre, 1986, 1989). The pathogen occurs worldwide in the temperate and subtropical zones in all continents, but only sporadically in the tropics.

The pathogen forms highly persistent survival structures, the microsclerotia, by which it may survive extended non-host periods. Given the wide host range of *V. dahliae*, crop rotation provides only limited protection against the disease, an alternation with monocotyledonous crops such as cereals yielding the best results (Bollen *et al.*, 1989). Control of verticillium wilt has largely been based on chemical soil disinfestation, but because of the nonselective mode of action and the environmental impact, such treatments are becoming banned by the authorities. Currently, alternatives are not available except for soil solarization in Mediterranean areas (Katan, 1981). Although microsclerotia are generally regarded as the target structures to be controlled through strategies of biological or cultural control, their ecology and dynamics have only rarely been studied in detail. Therefore, factors affecting formation, survival, and death of microsclerotia of *V. dahliae* are the subject of this thesis.

Besides *V. dahliae*, there are some other plant pathogenic species of *Verticillium*, i.e., *V. albo-atrum* Reinke & Berthold, *V. longisporum* (C. Stark) Karapapa, Bainbr. & Heale, and *V. tricorpus* Isaac. Their distinguishing morphological characters are listed in Table 1.2. *Verticillium* species are known to degenerate quickly after subculturing *in vitro* (Domsch *et al.*, 1980), so the identification should be performed using fresh isolates.

V. albo-atrum is known as the causal agent of wilt in mainly hop, tomato, soybean, and alfalfa (Domsch *et al.*, 1980; Atkinson, 1981). It is distinguished from *V. dahliae* by the absence of microsclerotia and the formation of dark resting mycelium, which has less survival potential than the mycelium of *V. dahliae*. The basal cells of conidiophores are usually dark-coloured. In contrast to *V. dahliae*, *V. albo-atrum* may become air-borne through the formation of conidia on infected shoot tissue (Jiménez-Díaz and Millar, 1988).

V. tricorpus is a weak pathogen that is able to produce three types of resting structures, i.e., resting mycelium, microsclerotia, and chlamydospores (Heale and Isaac, 1965). Korolev and Katan (1999) mentioned that although *V. tricorpus* colonized the roots and hypocotyls of seedlings, stems were only rarely infected. Paplomatas *et al.* (1997) reported preliminary evidence that *V. tricorpus* can be used as a biocontrol agent of *Rhizoctonia solani* in cotton seedlings.

V. longisporum was formerly named *V. dahliae* var. *longisporum* Stark (Stark, 1961), but studies on the morphology, phylogeny, and ecology revealed the necessity of recognition at the level of species (Jackson and Heale, 1985; Karapapa *et al.*, 1997). *V. longisporum* is recognised

Table 1.2. Morphologically distinguishing characters of the plant pathogenic *Verticillium* species (Isaac, 1953; Stark, 1961; Taylor, 1969; Domsch *et al.*, 1980; Karapapa *et al.*, 1997).

Characteristic	<i>V. albo-atrum</i>	<i>V. dahliae</i>	<i>V. longisporum</i>	<i>V. tricorpus</i>
Size of conidia (μm)	3.5-10.5(-12.5) \times 2-5	2.5-6(-8) \times 1.4-3.2	5-12.5 \times 1.6-3.5	3.5-10.0 \times 1.5-3.5
No. of phialides in terminal whorl	2-4	4-5	3	3-4
Resting structures	dark resting mycelium	microsclerotia	microsclerotia	dark resting mycelium, microsclerotia, chlamydospores
Shape of microsclerotia	-	elongate, sometimes spherical	elongate	elongated and connected to short, dark hyphae
Size of microsclerotia (μm)	-	50-200 \times 15-50(-100)	35-80	approx. 70-80

as a heterodiploid of *V. albo-atrum* \times *V. dahliae*. It can be distinguished morphologically from *V. dahliae* by three characters (Table 1.2), the most conspicuous is the longer conidia. In addition, phialides occur 3 per node in *V. longisporum* and usually 4-5 per node in *V. dahliae*. In contrast to *V. dahliae*, the host range of *V. longisporum* is limited to crucifers and the fungus has been most frequently reported to occur in oil seed rape (Berg, 1997; Karapapa *et al.*, 1997), though it was originally described from horseradish.

Three aspects are introduced here, viz., the aetiology and the life cycle of *V. dahliae*, the epidemiology of verticillium wilt diseases, and current control approaches for the pathogen, before the objective, approach, and structure of this thesis are outlined.

Life cycle of *Verticillium dahliae* and disease symptoms

The life cycle of *V. dahliae* can be divided into a dormant, parasitic, and saprotrophic stage (Figure 1.1). During the dormant stage the fungus survives in roundish to ellipsoid aggregates of thick-walled, melanized cells called microsclerotia. Melanins are dark-pigments found in animals, plants, and micro-organisms. They are not essential for growth or development (Polak, 1989), but provide protection against infection and, consequently, play an important role in the survival. Microsclerotia of *V. dahliae* have been shown to remain viable in soil for more than 10 years (Wilhelm, 1955; Powelson and Rowe, 1993). Hawke and Lazarovits (1995) found that non-melanized microsclerotia of a mutant had no persistence.

In soil, *V. dahliae* lacks a saprotrophic stage. Germination of microsclerotia, being subject to mycostasis (Lockwood, 1977), takes place only under the influence of root exudates in the rhizosphere of host plants. Also non-hosts have been reported to induce germination of microsclerotia (Schreiber and Green, 1963; Olsson and Nordbring-Hertz, 1985; Mol *et al.*, 1995). Mycostasis is probably overcome by root exudates in the rhizosphere where available carbon is not limited (Lynch and Whipps, 1990). Sites of infection of germinating microsclerotia are the root tip, the root elongation zone, or the points of lateral root emergence.

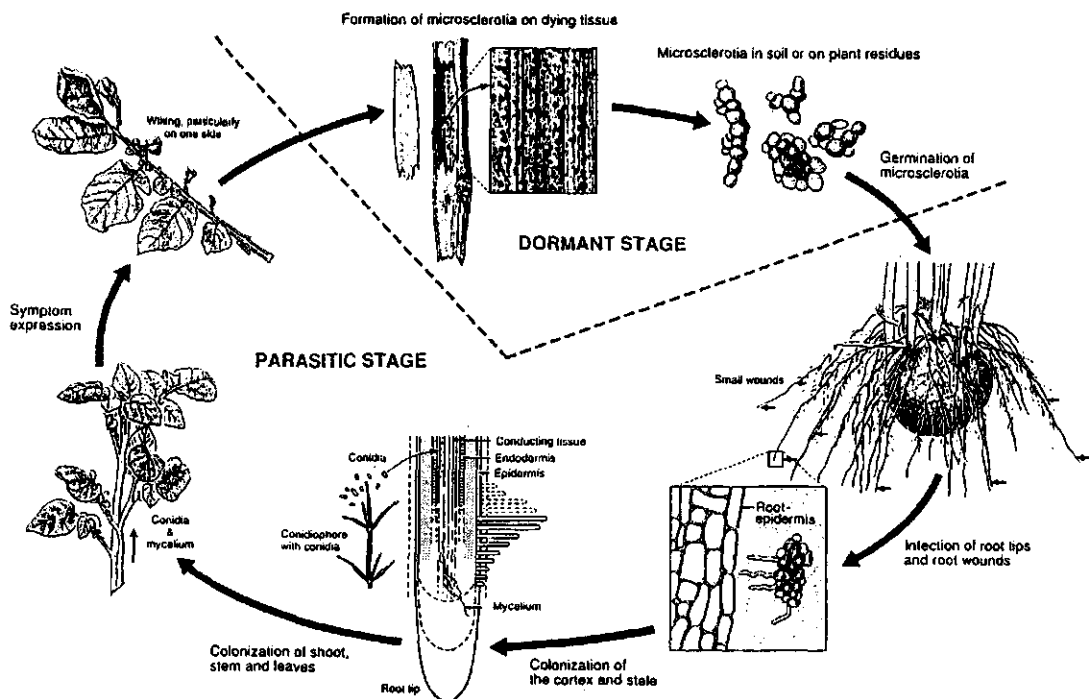


Figure 1.1. Life cycle of *Verticillium dahliae* in potato (Courtesy Dr M.P.M. Nagtzaam in Nagtzaam, 1998).

Most of the root cortex infections seem to affect the plant to a limited extent and they remain superficial and small (Huisman, 1988; Gerik and Huisman, 1988). In some plants, root infections remain limited in size probably as a result of a resistance reaction by the host. However, in chrysanthemum considerable damage was observed in infected root cells (Hall and Busch, 1971). The endodermis acts as a natural barrier that apparently cannot be penetrated by *V. dahliae*, only very few root cortex infections seem to reach the vascular tissue. Huisman (1982) estimated that only 5% or less of the root cortex infections ultimately results in infection of the shoot. It has been postulated that vascular infections are successful only where the endodermis has yet not developed (*i.e.*, at the root tip) (Bowers *et al.*, 1996), or where the endodermis has been damaged, *e.g.*, by nematodes (Pegg, 1974; Schnathorst, 1981; MacGuidwin and Rouse, 1990a).

After penetration of the endodermis, the fungus enters the vascular system where it forms conidia from inconspicuous intercalary phialides, also termed budding (Gerik and Huisman,

1985; Ferrandino, 1995). Subsequently, the conidia are transported passively with the sap stream to end walls where they germinate and penetrate into the next vessel segment (Bell, 1992), followed again by production of conidia. *V. dahliae* secretes pectinolytic enzymes that destroy the middle lamellae of the xylem parenchyma and degrade pectic compounds in the walls of vessels and tracheids (Pierson *et al.*, 1955; Heale and Gupta, 1972). Parenchyma cells adjacent to the infected xylem vessels become discoloured and filled with gum-like material. In most host species, xylem discoloration can be seen by the naked eye. Infected xylem vessels may become clogged by the secretion of material by the parenchyma cells and by masses of fungal hyphae. This, in combination with production of wilt toxins (*e.g.*, lipopolysaccharides) by the pathogen, results in reduced respiration, reduced photosynthesis, and, finally, wilting (Orenstein *et al.*, 1989; Mansoori *et al.*, 1995).

Wilt symptoms appear initially at the lower leaves, where, typically, first half of an infected leaf shows disease symptoms, but ultimately the whole leaf wilts and drops off. In many cases, however, no one-sided wilting of leaves occurs, and premature senescence is the only disease symptom. In trees, only some branches may show wilting and they may recover temporarily or completely in subsequent years (Hiemstra, 1998). Since specific disease symptoms are often absent, the disease is only diagnosed reliably when the pathogen can be isolated from diseased plant material. Moreover, several *Fusarium* species cause similar disease symptoms.

During and after plant senescence the saprotrophic stage of the fungus begins, resulting in the colonization of the shoots and roots followed by massive production of microsclerotia. Microsclerotia are formed most prominently in the cortex of stems, and more rarely in the leaves (cotton) of herbaceous plants. They are also commonly formed in petioles of woody hosts (Rijkers *et al.*, 1992).

Epidemiology of verticillium wilts

Verticillium wilt is considered to be a monocyclic disease, because a single propagule (*i.e.*, a microsclerotium) causes only one infection within one growing season and new microsclerotia may be formed in the season of infection, but they will not lead to new infection in the same season. A linear relationship between the density of microsclerotia in soil and the number of root infections per unit of root length has been reported frequently (Huisman and Ashworth, 1976; Ashworth *et al.*, 1979; Nagtzaam *et al.*, 1997). *V. dahliae* may be considered an ecologically obligate vascular pathogen. A short growth through soil to reach a root is possibly at the expense

of the energy contents of the microsclerotia.

It has been shown that microsclerotia may germinate independently of an external nutrient supply (Ben-Yephet and Pinkas, 1977). However, as described in the previous paragraph, in an unsterile soil, *V. dahliae* is subject to the phenomenon of mycostasis. Lockwood (1977) hypothesized that propagules can germinate by consuming exudates accumulated on their surface. In an unsterile soil, these exudates are removed by other organisms, thus preventing germination. Mycostasis has been interpreted as ecologically advantageous for any soil-borne plant pathogen because in the absence of a host their germination hyphae would be lysed readily. In the rhizosphere, however, where through the exudation of nutrients by the root carbon limitation is counteracted (Lynch and Whipps, 1990), microsclerotia of *V. dahliae* germinate readily (Mol and Van Riesen, 1995). The production of new microsclerotia from germinated microsclerotia has also been reported (Farley *et al.*, 1971), but this new microsclerotium receives less energy than the original one, as the uptake of nutrients from the surrounding soil is considered to be highly unlikely. The energy contents of a microsclerotium are likely to be sufficient to support hyphal growth over a very short distance towards a root.

Each single cell of a microsclerotium is able to germinate once. Thus, microsclerotia are able to germinate repeatedly. A linear relationship has been observed between density of microsclerotia in soil and root infection or disease incidence (Ashworth *et al.*, 1979; Nagtzaam *et al.*, 1997). However, in several other studies no significant correlation was found between microsclerotial density in soil and infection or yield loss (Ashworth *et al.*, 1972; DeVay *et al.*, 1974; Davis and Everson, 1986; Bejarano-Alcázar *et al.*, 1995). Some dispersal of *V. dahliae* through the soil is possible by mesofauna or abiotic factors. Uneven distribution of the microsclerotia may also play a role. Bejarano-Alcázar *et al.* (1999) observed infection in all parts of the root systems of eggplants that were inoculated by placing about 100 microsclerotia adhering to a 9-18 mm²-sized tape against the root for three days. They suggested that production of conidia on microsclerotia and subsequent transport of these conidia by water flow or the mesofauna may explain the distribution of infection on the eggplant root system. Transmission of conidia of *Coniothyrium minitans* in soil by the mesofauna has been shown by Williams and Whipps (1995). However, for time-spans longer than a few weeks, microsclerotia are the only survival structures of *V. dahliae* in soil (Green, 1969).

The main mechanisms of dispersal of *V. dahliae* are probably through transport of infested soil by agricultural tools or by the wind, by using infected plant material or by infested soil adhering to plant material. Rowe *et al.* (1997) showed a high incidence of infection in potato seed tubers. Seed transmission seems to be an important mechanism of dispersal of *V. dahliae* which

has been demonstrated for safflower (Klisiewicz, 1975), sunflower (Sackston and Martens, 1959; Richardson, 1990), cotton, eggplant, linseed, soybean, and tomato (Richardson, 1990), and spinach and beet (Van der Spek, 1972).

Genetic variation of *V. dahliae*, presence of root-infecting nematodes and environmental conditions may explain differences in the relation between soil inoculum density and disease incidence among fields. Although *V. dahliae* has long been regarded as a fairly homogeneous species, several pathotypes and, more recently, four or five vegetative compatibility groups have been recognised (Jeger *et al.*, 1996). These vegetative compatibility groups exhibit some specialization with respect to host range, aggressiveness, and distribution. For example, vegetative compatibility group 4A is most commonly encountered in solanaceous crops in Europe and America (Joaquim and Rowe, 1991). Of course the virulence of particular isolates of *V. dahliae* depends also on the susceptibility of the host cultivar (Zilberstein *et al.*, 1983).

Plant-pathogenic nematodes are known to enhance verticillium wilt severity (*e.g.*, Harrison, 1971; MacGuidwin and Rouse, 1990ab; Bowers *et al.*, 1996). The mechanism of this interaction is not well-understood. Both direct (provision of infection courts for *V. dahliae* by root wounding caused by nematodes; Mai and Abawi, 1987) and indirect interactions (increased susceptibility; Faulkner *et al.*, 1970) have been suggested. The interaction between verticillium wilt and root-infecting nematodes is likely to be of considerable importance, given the wide host ranges and strong survival capabilities of both nematodes and *V. dahliae*.

Moderately high temperatures favour yield loss in potato (Francel *et al.*, 1990). However, supra-optimal temperatures curtail further development of *V. dahliae*. In potato-growing areas disease development of *V. dahliae* is limited to the spring and autumn, because temperatures become too high in summer (Tsrur *et al.*, 1990). Germination of conidia and mycelial growth at temperatures above 31-33°C (Pullman *et al.*, 1981) and the development of *V. dahliae* in infected plants stop at temperatures above 27°C (Rowe *et al.*, 1987; Koike *et al.*, 1994). This may be the major reason that *V. dahliae* is of less importance in tropical regions. Excessive soil moisture is known to enhance potato early dying in irrigated areas with an arid climate. This may be caused by a fast transpiration stream (Cook, 1973), although several other factors such as effects on root growth and root exudation have also been proposed (Pullman and DeVay, 1982; Gaudreault *et al.*, 1995). In more temperate areas the effect of soil moisture is, however, less evident (Bollen *et al.*, 1989; Haverkort *et al.*, 1990).

Yield reduction is mainly the result of blockage of the xylem vessels, closure of the stomata, reduced photosynthesis, and early senescence (Haverkort *et al.*, 1990; Bowden and Rouse, 1991; Schnathorst, 1981). The production of toxins by *V. dahliae* possibly also

contributes to yield reduction (Heale and Gupta, 1972; Orenstein *et al.*, 1989).

New microsclerotia are formed when infected plant parts senesce. *V. dahliae* leaves the xylem, colonizes the parenchymatous tissues and forms microsclerotia. When plant parts bearing microsclerotia are incorporated into the soil, aggregated microsclerotia are initially held together by the shoot tissue, but gradually, as the shoot decomposes, the microsclerotia fall apart. This is reflected in an apparent increase in soil inoculum density one or two years after the incorporation of infected plant material (Mol *et al.*, 1996a). Wilhelm (1955) reported that microsclerotia may survive in soil for 12-14 years. Populations of microsclerotia gradually decrease with time down to levels where damage to the host is insignificant. Bollen *et al.* (1989) found that growing non-hosts for four years was sufficient to eliminate damage to *V. dahliae* in potato. The effects of environmental conditions on the survival of microsclerotia have been studied only rarely. Artificially produced microsclerotia may have different survival patterns from those formed naturally. In unsterile soil, they survived only up to 60 days (Lazarovits *et al.*, 1991).

Moist conditions resulting from heavy rains and flooding of potato fields were found to induce production of microsclerotia of *V. dahliae* on the surface of moribund stems and leaves after plant senescence (McKeen and Thorpe, 1981; Stapleton *et al.*, 1993). *V. dahliae*, however, also can grow, sporulate, and form microsclerotia under dry conditions of -100 to -120 bars (approx. pF 5) (Ioannou *et al.*, 1977a). Further Lazarovits *et al.* (1991) reported that microsclerotia in water-saturated soils lost viability more quickly (less than 10 hours) than in soils that were air-dry or at 50% of field capacity. Mol and Scholte (1995b) found that covering the plant remains with soil did not reduce the production of microsclerotia. Root colonization by *V. dahliae* was suppressed consistently by a soil water tension of -0.01 MPa (pF 2) (Gaudreault *et al.*, 1995). The findings of Ioannou *et al.* (1977a) and Powelson and Rowe (1993) that early-season irrigation can be an effective component of integrated disease management support this.

Management of verticillium wilt disease

Many non-hosts, including various monocotyledonous crops, can be symptomless carriers of *V. dahliae*. Attempts have been made to reduce inoculum densities of *V. dahliae* by inducing germination of microsclerotia by non-hosts. However, new microsclerotia were produced in the roots of non-hosts, resulting in insufficient control of *V. dahliae* (Mol *et al.*, 1996a).

Breeding for resistance or tolerance has been successful for only a few crops. Commercial resistant or tolerant crops are only available in tomato and cotton production (Allen, 1994) and in

sunflower (Miller and Gulya, 1995). Tolerance is available in other crops such as potato, but presently the choice of potato cultivars is dominated by the requirements of the processing industry and preferences of the consumers.

Crop rotation is commonly recommended as a method to control *V. dahliae*. Cropping non-hosts for four years or more markedly reduces populations of *V. dahliae* (Bollen *et al.*, 1989). However, given the wide host range of *V. dahliae*, non-hosts may not be an economic alternative to the farmer. Moreover, crops such as cotton and potato are grown continuously in various areas. Under these circumstances soil steaming or fumigation has been applied. These methods, however, become more and more restricted due to high costs, health risk for workers, and adverse effects on the environment.

Soil solarization provides control in areas with a Mediterranean climate (Katan, 1980). Soil inundation may also be effective provided that low oxygen levels develop and temperatures are not too low (Ioannou *et al.*, 1977b). In the Netherlands, Blok *et al.* (2000) obtained at least 90% reduction of inoculum density of *V. dahliae* by inducing anoxic conditions through the incorporation of fresh organic matter and overnight irrigation followed by the placement of a plastic tarp. In addition to the direct effect on the fungus, nematodes stimulating verticillium wilt were also controlled. Conn and Lazarovits (1998) were able to reduce verticillium by the application of chicken manure. Other organic amendments that showed an effect on *V. dahliae* are alfalfa and oat residues (Green and Papavizas, 1968), chitin (Jordan *et al.*, 1972) or barley (Harrison, 1976). Davis *et al.* (1996) concluded that green manure treatments with sudangrass or maize gave best control of verticillium wilt. Koike *et al.* (1997) and Subbarao *et al.* (1999) demonstrated the effectiveness of broccoli residues to control verticillium wilt in cauliflower. The removal of debris containing microsclerotia seems a logical approach to control *V. dahliae* but, according to Mol *et al.* (1995), this treatment was insufficient to allow narrow rotations of potato.

Soils that are suppressive to *V. dahliae* are barely reported. Ashworth *et al.* (1976) showed that Cu^{2+} was involved in the suppression of *V. dahliae*. This effect was noticed when no microsclerotia were recovered from soils which previously had contained high densities of them. Further data on this phenomenon have not been reported. Keinath and Fravel (1992) were able to induce suppressiveness in the greenhouse by repeatedly cropping potato on the same soil. The induction of this suppressiveness varied with soil type, but its nature was not further investigated. The phenomenon of disease suppression, which is understood in several other soilborne pathosystems (e.g., Baker and Cook, 1974; Oyarzun *et al.*, 1997, 1998) is still not explained for verticillium.

The potential of the application of biocontrol agents to control verticillium wilt is now being studied intensively. About 25 potential antagonists have been tested. In most, if not all, cases, these antagonists have been selected from studies on other sclerotial parasites. The study of parasites of microsclerotia is hampered by their small size. Recently investigated antagonists include *Trichoderma* (*Gliocladium*) *virens* (Johnston *et al.*, 1994), *Trichoderma koningii* (Georgieva, 1992) and other *Trichoderma* species, *Bacillus subtilis*, *Pseudomonas fluorescens*, and *Streptomyces flavofungini* (Berg and Ballin, 1994), *Pythium oligandrum* (Al-Rawahi and Hancock, 1998) and, most notably, *Talaromyces flavus* (Marois *et al.*, 1982; Fravel, 1996). *T. flavus* is able to parasitize microsclerotia of *V. dahliae* (Fahima *et al.*, 1992) and sclerotia of several other pathogens such as *Sclerotinia sclerotiorum* (McLaren *et al.*, 1986), *Rhizoctonia solani* (Boosalis, 1956), and *Sclerotium rolfsii* (Madi *et al.*, 1992). Furthermore, *T. flavus* is able to produce enzymes such as glucose oxidase that generates toxic peroxide, chitinase, glucanase, and cellulase. Madi *et al.* (1997) showed that culture filtrates of *T. flavus* affected germination and melanin formation of microsclerotia. McLaren *et al.* (1986) reported partial disintegration of melanized material of sclerotia of *S. sclerotiorum* that were parasitised by *T. flavus*. Although *T. flavus* is regarded as a root colonizer, the factors affecting the population dynamics in the rhizosphere are insufficiently understood (Fahima and Henis, 1995; Nagtzaam and Bollen, 1997). Recently *T. flavus* has been registered as a biocontrol agent by Prophyta (Rostock, Germany) for the control in oil-seed rape and tomato. Both root dipping of young plants or mixing of the suspended product is advised (Zeise, 1997; Koch, 1999). So far, application of *T. flavus* to the soil has yielded inconsistent results, probably because of competition with the resident microflora.

About this thesis

The main goal of the present study was to gather fundamental ecological knowledge on the formation and survival of microsclerotia. Detailed quantitative information about factors influencing these processes may lead to new prospects for controlling *V. dahliae*. A secondary goal was to further improve biological control of verticillium wilt. We hypothesized that the above-ground application of the biocontrol agent *Talaromyces flavus* onto microsclerotia-containing debris would lead to reduction of inoculum density of *V. dahliae*. In addition, the effects of a diacetylphloroglucinol-producing strain of *Pseudomonas fluorescens* on verticillium wilt was studied.

In Chapter 2 a bioassay is presented for the quantitative study of the formation of microsclerotia and for evaluating the effects of biocontrol agents using *Arabidopsis thaliana* as a test plant. *A. thaliana* was chosen because of its short life cycle and its sensitivity to many plant pathogens. Many other bioassay plants require either labour-intensive methods, involving the plating of root or shoot fragments to detect or quantify *V. dahliae*, or they need a long period before disease symptoms develop, or do not allow to detect low population levels of, e.g., 1 microsclerotium per gramme soil, that may still damage a susceptible crop.

In Chapter 3 the effects of constant and variable temperatures on the formation of microsclerotia on *A. thaliana* are assessed. In the field a large variation in density of microsclerotia is commonly observed in senescent host tissue. We studied whether temperature affects the process of microsclerotium formation, either directly or indirectly through plant senescence.

In Chapter 4 factors affecting the density of microsclerotia distributed in unsterile soil are investigated. Factors studied include temperature, moisture, and different methods of incorporation of organic debris.

Chapter 5 studies effects of various methods of application of the antagonist *T. flavus* on survival of the pathogen on potato stems covered with microsclerotia of *V. dahliae*.

In Chapter 6 the effects of a 2,4-diacetylphloroglucinol-producing strain of *P. fluorescens* on verticillium wilt in *A. thaliana* and eggplant, with or without in combination with *T. flavus* are reported.

Finally, in Chapter 7 topics of bioassays, formation and survival of microsclerotia, and biocontrol are discussed in a more general context.

CHAPTER 2

ARABIDOPSIS THALIANA AS A BIOASSAY PLANT FOR VERTICILLIUM DAHLIAE

Summary

Arabidopsis thaliana was used as a bioassay plant to detect low inoculum levels of *Verticillium dahliae* in soil. Although in the field crucifers are predominantly infected by *V. longisporum*, under the test conditions *A. thaliana* became readily infected by four isolates of *V. dahliae*. Disease symptoms included early senescence, but no typical verticillium wilt. Increasing amounts of infection of both root and shoot were noted at densities of 1, 3, 10, 30, and 100 microsclerotia g⁻¹ soil. A log-linear relation between inoculum density and the area-under-the-disease-progress curve was established. Even at the lowest inoculum density of 1 microsclerotium g⁻¹ soil, 5% of the root length was infected with *V. dahliae*, and 30% of the stems. The most sensitive parameter was the density of new microsclerotia formed in the shoot. The higher the inoculum density the greater the density of microsclerotia in the shoot, indicating that multiple root infections lead to a more intense colonization of *V. dahliae*. Disease progress was fastest at 20°C, and slower at 10, 15, and 25°C. A bioassay with *A. thaliana* for assessing infestation of fields with *V. dahliae* can be recommended.

Introduction

Verticillium dahliae Kleb. causes considerable economic losses through wilting and early senescence in diverse crops such as potato, cotton, rose, strawberry, and maple (Schnathorst, 1981). This root-infecting pathogen forms abundant survival structures, microsclerotia, in diseased, senescent tissue. Given the wide host range of *V. dahliae*, crop rotation provides only limited protection against the disease, an alternation with monocotyledonous crops such as cereals giving the best results (Bollen *et al.*, 1989). Control of *V. dahliae* has largely been based on the application of chemical soil disinfectants, but because of their impact on the soil

ecosystem, the groundwater quality, and the contribution to the depletion of the ozonosphere by methyl bromide, these methods are becoming unacceptable.

For the development and evaluation of new management strategies against *V. dahliae*, assays are needed that, rapidly and with limited labour input, provide information about the density of *V. dahliae* microsclerotia in soil to evaluate the need for control or control efficacy. Changes in the soil inoculum density of *V. dahliae* are often assessed by plating a soil suspension onto a semi-selective agar medium, but these determinations are not always reliable (Termorshuizen *et al.*, 1998). Moreover, they exclude effects of competition in the rhizosphere and of induced resistance. Therefore, bioassays have been recommended to study the ecology of *V. dahliae* (e.g., Evans *et al.*, 1974), for selection of resistance to the pathogen (Palloix *et al.*, 1990) and for the evaluation of biocontrol agents (e.g., Nagtzaam *et al.*, 1998). However, bioassays so far available are either labour-intensive, involving plating of root or shoot fragments to detect or quantify *V. dahliae*, or disease symptoms take a long time to develop. Moreover, presently available bioassays are not able to detect low population levels of e.g. 1 microsclerotium per gramme soil that may still damage a susceptible crop (Nicot and Rouse, 1987).

Arabidopsis thaliana (L.) Heyhn. having an unusually small genome among green plants has become a guinea pig for studies on gene expression. In preliminary experiments, at this institute, *A. thaliana* was found to be sensitive to *V. dahliae*. We therefore decided to try its use as a test plant in bioassays for *V. dahliae*. *A. thaliana* is used in many studies, with fungi (Koch and Slusarenko, 1990), plant-pathogenic bacteria (Whalen *et al.*, 1991; Tsuji and Somerville, 1992), nematodes (Sijmons *et al.*, 1991), and viruses (Sosnova and Polak, 1975), in part because of its short life cycle. Cruciferous crops such as oil seed rape are long known to be preferentially infected by heterodiploid strains of *V. dahliae* formerly known as *V. dahliae* var. *longisporum*, but recently this taxon was elevated to species level (Karapapa *et al.*, 1997). Tabrett *et al.* (1995), however, showed that the haploid *V. dahliae* also was able to infect the crucifer *A. thaliana*.

Material and methods

Fungal collections, maintenance, and inoculum preparation

Four collections of *V. dahliae*, K3 and V1, V3, and V4, were obtained in summer 1996 and 1997, respectively, from diseased potato stems containing microsclerotia in the municipalities of Veenhuizen, Nieuweroord, Nieuw-Balinge, and Bruntinge, respectively, in the Province of

Drenthe, the Netherlands. The potato stems were cut into 1-cm pieces, allowed to air dry for about ten days at room temperature, and stored in plastic boxes until further use.

To separate the microsclerotia from potato stem tissue the material was ground and sieved over screens with mesh sizes of 106 and 20 μm . The residue remaining on the 20 μm sieve was suspended in water containing 0.08% agar. The agar was added to obtain a homogeneous suspension of microsclerotia. The number of microsclerotia in the suspension was determined in small subsamples using a dissecting microscope at 25 \times magnification. To determine the germinability of microsclerotia, 25 microsclerotia were plated individually in Petri dishes containing Modified Soil Extract Agar (MSEA) medium (Harris *et al.*, 1993) by using an insect pin 000 (Emil Arlt, Australia). The Petri dishes were incubated upside down in the dark for 14 days at 23°C, and the number of germinated microsclerotia was counted.

Microsclerotia from collections V1, V3, and V4 were surface-disinfested in 1% NaClO for 1 min, washed three times in sterile water each for 1 min, blotted dry on sterile filter paper, and plated onto Potato-Dextrose Agar (Oxoid) amended with 50 ppm oxytetracyclin. From this culture monoconidial isolates were prepared. This culture was propagated by growing in Czapek-Dox liquid medium (Oxoid) for 7 days at 23°C in a shaking incubator (Gallenkamp Orbital Incubator). After incubation, conidia were filtered through cheese cloth and suspended in sterile distilled water to obtain 1.0×10^6 conidia ml^{-1} suspension.

Plant material and growth conditions

A. thaliana ecotype Columbia was used in this study. A preliminary experiment did not indicate significant differences in colonization with three other ecotypes of *A. thaliana*, i.e., Cape Verde Islands, Landsberg *erecta*, and Wassilewskaija. Seeds of *A. thaliana* were provided by Prof. Dr M. Koornneef (Laboratory of Genetics, Wageningen University). Seeds were spread over sieved and wetted potting soil in a plastic box covered with a cap in order to maintain high humidity. The boxes were incubated in a climate chamber with 16 h light (Philips TD32W/84 HF) and 8 h dark at 20°C.

To determine infection by *V. dahliae*, *A. thaliana* was harvested after its rosette had completely died. Roots were washed on a sieve by spraying tap water to remove organic matter. Stem segments were surface-sterilized in 1% NaClO for 30 sec, root segments in 96% ethanol for 1 sec, then washed twice in sterile water, dried on sterile filter paper, plated separately in Petri dishes containing MSEA and incubated at 25°C in the dark for 14 days.

Disease assessment

Senescence of rosette leaves and stems (including stems, stem leaves, flowers and siliquae) was evaluated separately by assessing senescence indices (SI) ranging from 0 to 5 in weekly intervals. For the rosette leaves: 0 = 0-5% of the rosette leaves showing some necrosis; 1 = 6-15%; 2 = 16-25%; 3 = 26-50%; 4 = 51-75%; and 5 = 76-100%. For the stems: 0 = no necrosis; 1 = stems still green but 1-5% of leaves showing necrosis; 2 = 1-5% of the stem brownish or yellowish, but 6-25% of the leaves showing necrosis, and 1-5% of pods and/or flowers yellowish or brownish; 3 = 6-25% of the stems brownish or yellowish, 26-75% of the leaves showing necrosis, and 6-25% of the pods brownish; 4 = 26-50% of the stems brown, 26-100% of the leaves showing necrosis, 26-50% of the pods brown and 1-75% of the flowers yellow or brown; and 5 = most of all stems, leaves, flowers, and pods show necrosis. The disease index (DI) of verticillium-inoculated plants was defined as SI after subtraction of the values of SI determined for the control plants. Using these indices, the area-under-the-senescence-progress curve (AUSPC) and the area-under-the-disease-progress curve (AUDPC) were calculated (Campbell and Madden, 1990).

Production of microsclerotia of *V. dahliae* was quantified after *A. thaliana* had completely died. Shoots and roots were separated and the roots were washed by gently soaking in standing tap water. All parts of the plant were then dried at room temperature for a week. Both dried shoots and roots of *A. thaliana* were weighed and ground using a mortar and pestle and suspended in 0.08% water agar. The suspension was weighed and homogenized. The density of microsclerotia in the suspension was determined by direct counting of at least three small subsamples under the dissecting microscope at 16 × magnification measuring approximately 20 µl.

Experiments

Effect of inoculum density on disease development. The experiment was carried out as a completely randomized block design using densities of 0, 1, 3, 10, 30, and 100 microsclerotia g⁻¹ dry soil and was performed with 10 replications per treatment. Plastic pots (w/w/h, 7/7/8 cm) were used containing mixed sieved potting soil and sand (2/1, v/v). Four-week-old seedlings of *A. thaliana* with the first rosette leaf emerging were planted and placed in a climate chamber at 25°C under a 16/8 h light/dark regime. A repeated experiment showed similar results, and only those of the first experiment are presented here.

Effects of temperature. The optimal bioassay temperature and the pathogenicity of three isolates of *V. dahliae* were determined in a root-dip experiment with isolates V1, V3, and V4. Four-week-

old seedlings of *A. thaliana* were uprooted carefully and the roots were washed in sterile distilled water, dipped for 20 min in a conidial suspension of *V. dahliae* (10^6 conidia ml⁻¹), and planted in pots with mixed potting soil and sand as described above. The pots were placed at 10, 15, 20, and 25°C, under the same light regime.

Results

Disease symptoms became visible as early as 4 weeks after planting the seedlings of *A. thaliana* in infested soil or dipping the seedlings in a conidial suspension. Symptoms consisted of chlorosis followed by necrosis, sometimes accompanied by some stunting. The typical unilateral wilting of leaves or plants, often described for verticillium wilt was not observed. Nine weeks after planting, most rosette leaves were completely dead and 11 weeks after planting the stems had nearly or completely died. During the final stage of senescence, microsclerotia were formed abundantly, especially on the stems, but many were also observed on all other plant parts, including the root system. The dry weight of the shoot was not significantly affected by *V. dahliae*. Natural senescence progressed slowly at low temperatures. Senescence due to *V. dahliae* was most strongly enhanced at 20°C but less at 15 and 25°C (Table 2.1).

Senescence began earlier and progressed faster at increasing densities of microsclerotia in soil (Figure 2.1). Since senescence is a rapid natural process in *A. thaliana* all plants eventually became senescent; thus differences in senescence between treatments tend to converge at the end

Table 2.1. The time (days) it takes for the plants to die of seedlings that were root-dip infected with three isolates of *Verticillium dahliae* (10^6 cfu ml⁻¹ suspension) to uninfected control plants.

Temperature (°C)	Isolate of <i>Verticillium dahliae</i>		
	V1	V3	V4
	Life span of <i>Arabidopsis thaliana</i> relative to the control ¹		
10	0.81	0.86	0.82
15	0.82	0.80	0.79
20	0.66	0.59	0.66
25	0.76	0.71	0.71

¹Control plants had life spans of 293, 247, 178, and 99 days for incubation temperatures of 10, 15, 20, and 25°C respectively.

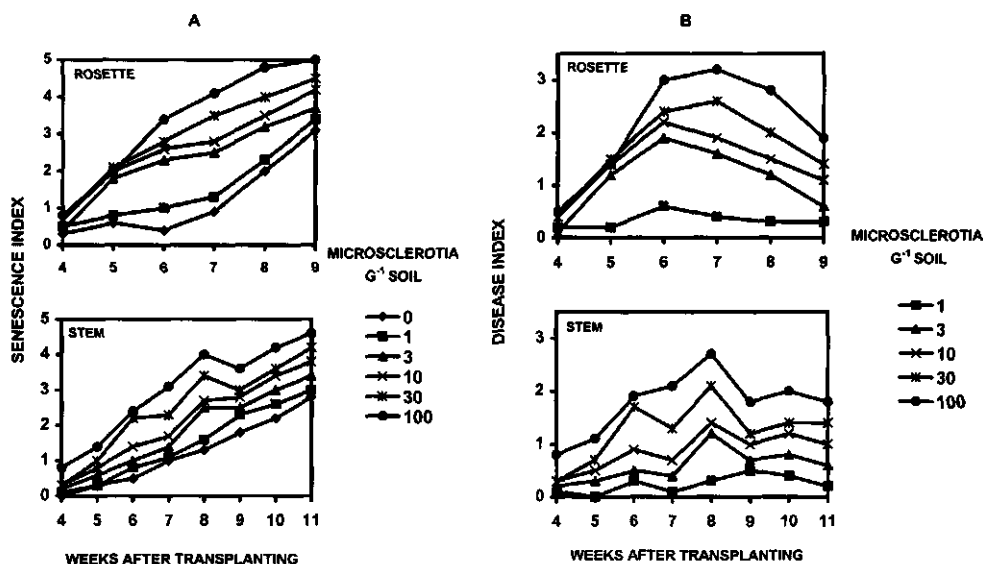


Figure 2.1. Senescence progress for rosette leaves and stems of *Arabidopsis thaliana* at different inoculum densities of *Verticillium dahliae*. (A) Senescence index, (B) Disease index = senescence index minus the senescence index estimated for the control plants.

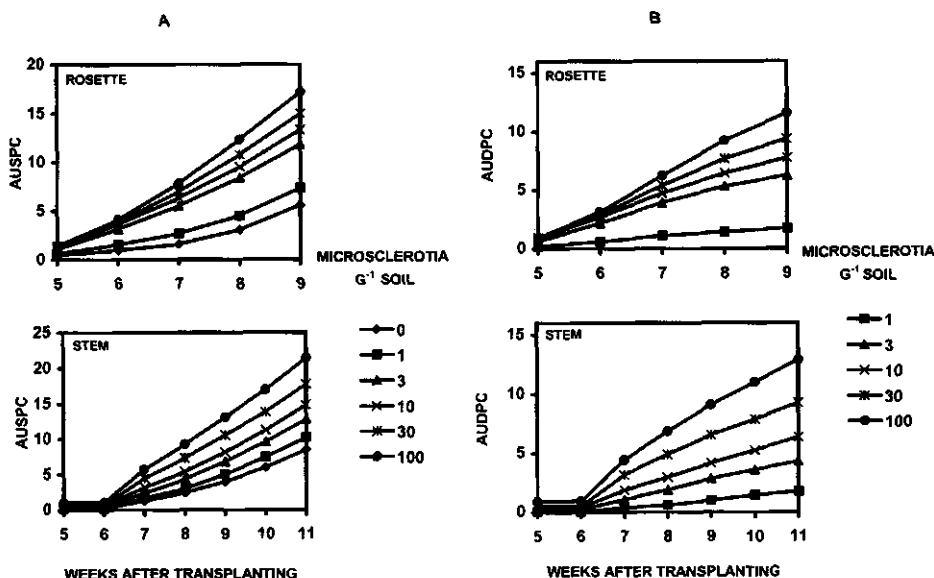


Figure 2.2. Senescence progress for rosette leaves and stems of *Arabidopsis thaliana* at different inoculum densities of *Verticillium dahliae*. (A) Area-under-the-senescence-progress curve (AUSPC), (B) Area-under-the-disease-progress curve (AUDPC) = AUSPC minus AUSPC estimated for the control plants.

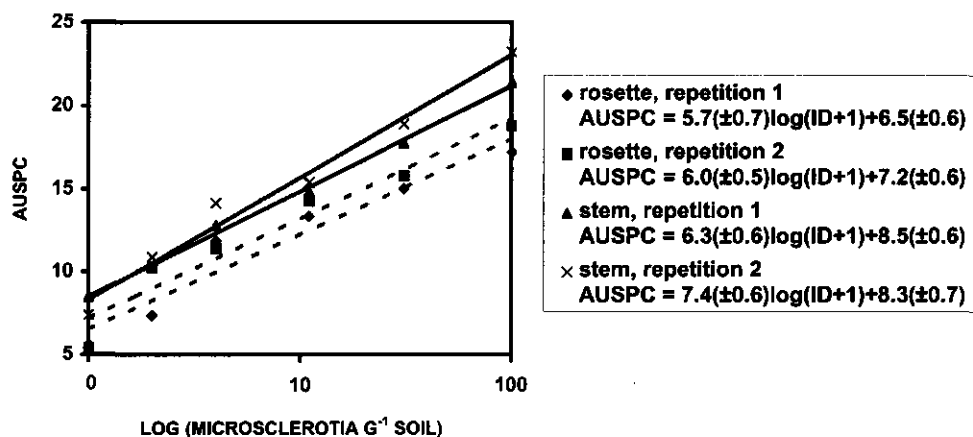


Figure 2.3. Regression lines for the area-under-the-senescence-progress curve (AUSPC) as a function of the transformed ($^{10}\log$) soil inoculum density.

of the life cycle of *A. thaliana* (Figure 2.1). The disease index (*i.e.*, the senescence index scored for the inoculated seedlings minus the senescence index scored for the non-inoculated seedlings) was maximal 6-8 weeks after transplanting the seedlings into infested soil for the rosette leaves, and 6-9 weeks for the stems (Figure 2.1). Disease indices for the rosette leaves generally gave less variable results than those for the stems. In several cases disease indices decreased temporarily for the estimates based on the stems, perhaps indicating that compensation growth had occurred. The area-under-the-senescence-progress curves steadily increased and showed the largest differences at the final observation date (Figure 2.2). The area-under-the-disease-progress curves showed few differences with the area-under-the-senescence-progress curves (Figure 2.2). At the latest observation date (9 and 11 week after transplanting for the rosette leaves and stems respectively), a log-linear relationship existed between inoculum density and the area-under-the-senescence-progress curve (Figure 2.3), with adjusted- R^2 values close to 0.70. At harvest, 11 weeks after transplanting, plating of the stem bases of seedlings that had been inoculated at 1 microsclerotium g^{-1} soil resulted in 30% incidence of *V. dahliae* (Figure 2.4). Individual colonies growing out from the roots could not be counted because most root infections appeared to be systemic. Therefore, the percentage of root length covered with *V. dahliae* after 14 days of incubation on MSEA was determined, resulting in 5% root infection at the lowest inoculum level. Infection gradually increased with increasing inoculum levels, leading up to 100 for stem and 70% for root infection, at 100 microsclerotia g^{-1} soil (Figure 2.4).

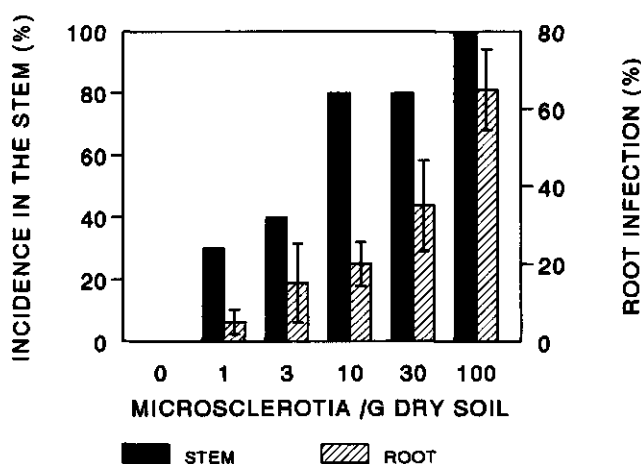


Figure 2.4. Incidence of *Verticillium dahliae* in the stem base and percentage of root length infected of completely senescent *Arabidopsis thaliana* as revealed by plating in relation to soil inoculum density.

The numbers of microsclerotia g^{-1} dry shoot tissue were correlated with the inoculum density of *V. dahliae*. While at 1 microsclerotia g^{-1} soil $1.6 \times 10^3 \pm 9.0 \times 10^2$ microsclerotia g^{-1} dry shoot tissue was found, at 100 microsclerotia g^{-1} soil an 18-fold increase was observed (Figure 2.5). Irrespective of the inoculum level, incidence of microsclerotia in the shoots was 100%. However, not all the stems produced by a single test plant contained externally visible microsclerotia (Figure 2.5); the proportion of stems with externally visible microsclerotia was strongly correlated with inoculum density.

Discussion

The four isolates tested had conidial lengths up to $6.0 \mu\text{m}$, indicating that they belong to *V. dahliae*, and not to *V. longisporum*, which has conidia up to $8.8 \mu\text{m}$ (Karapapa *et al.*, 1997). Problems with verticillium wilt in oil seed rape (*Brassica napus* var. *oleifera*) and other crucifers have been associated with *V. longisporum*, and mostly not *V. dahliae*, whilst on non-crucifers they appear to be always associated with *V. dahliae*, and not *V. longisporum* (Okoli *et al.*, 1994; Subbarao *et al.*, 1995). However, when inoculating *V. dahliae* or *V. longisporum* on crucifer or non-crucifer hosts, disease regularly occurred (Subbarao *et al.*, 1995; Tabrett *et al.*, 1995;

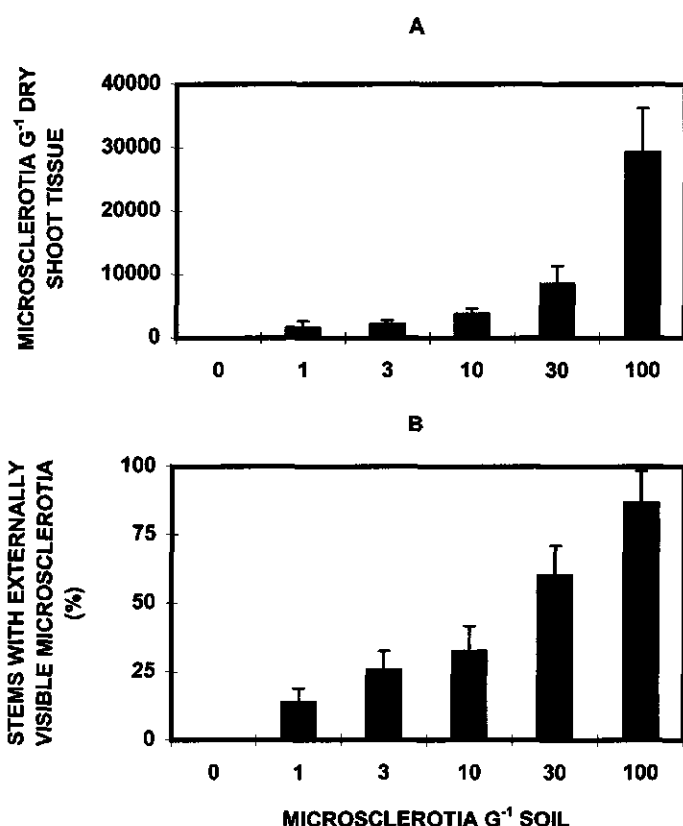


Figure 2.5. Microsclerotia in shoot tissue after complete senescence of *Arabidopsis thaliana* as a function of soil inoculum density. (A) The amount of microsclerotia g⁻¹ dry shoot tissue, (B) The percentage of stems per plant exhibiting externally visible microsclerotia.

Karapapa *et al.*, 1997). Apparently, in the field other mechanisms are operative that suppress disease development or dispersal of the pathogen in *V. dahliae* / crucifer and *V. longisporum* / non-crucifer combinations.

A. thaliana was susceptible to all four isolates of *V. dahliae* used in this study. Each isolate caused similar disease symptoms and formed microsclerotia in all parts of the senescent plants. However, typical disease symptoms of verticillium wilt, such as partial wilting of the leaves, were not observed. All parameters measured, except shoot dry weight, showed strong positive correlations with soil inoculum density of *V. dahliae*. Even at 1 microsclerotium g⁻¹ soil, stem and root infection was detected and the area-under-the-senescence-progress curve differed significantly from that of the control plants. The sensitivity of detecting *V. dahliae* by plating stem base fragments appear to be lower than that of assessing microsclerotia in the shoot,

senescence index, or root plating. For example, microsclerotia were detected in all stems of plants exposed to 1 microsclerotium g⁻¹ soil, although only 30% of them showed presence of *V. dahliae* on plated stem base fragments. Nagtzaam *et al.* (1997) found that plating of 5-mm pieces of stem bases of eggplant or potato was much less sensitive than plating sap squeezed from 5-cm stem pieces. This indicates either that low populations of *V. dahliae* are unable to grow out from the stem pieces onto the agar medium or that the pathogen may simply be absent from the small stem pieces. Using ELISA and plating, Van der Koppel and Schots (1995) mentioned uneven distributions of *V. dahliae* in stems of various hosts. Likewise, in our platings of the 5-cm-long pieces of the stem base of *A. thaliana*, *V. dahliae* may have been absent, while being present in other parts of the stem.

The association of *V. dahliae* with the crucifer *A. thaliana* may be rare under field conditions, if extant at all, but for bioassay purposes it appears to be well-suited. The bioassay is relatively fast as already 6 weeks after planting final disease scores can be made. The bioassay appears to be suited to detect low levels of soil infestation with *V. dahliae*. Assessment of disease 6-9 weeks after planting is optimal when the differences with the control are most prominent. The disease index is the parameter most easily determined. More sharply defined criteria include the number of isolations of *V. dahliae* obtained from the shoot or root, and the amount of microsclerotia formed on the shoot after complete senescence of the plants. Instead of determining the amount of microsclerotia per unit of dry weight of shoot material, a semi-quantitative index for microsclerotial density may also be used (data not presented). However, we prefer the use of disease indices because they discriminate sufficiently between the different inoculum densities. *A. thaliana* responded more sensitively to low inoculum densities than other bioassay plants such as eggplant (Nagtzaam *et al.*, 1997) or thorn apple (Evans *et al.*, 1974). For example, at several (but not all) observation dates 5-7 weeks after planting, disease indices differed significantly ($P < 0.05$) between inoculum densities 0, 1, and 3 microsclerotia per gramme dry soil. Disease progressed most quickly in plants grown at 25°C, but the life span of the *V. dahliae*-infected plants was most influenced at 20°C (Table 2.1). Therefore, we recommend a temperature of 20°C for the bioassay.

In all test plants, the proportion of stems exhibiting externally visible microsclerotia was strongly positively correlated with inoculum density. This may indicate that a single infection on a root is not able to infect all stems of a plant. The amount of microsclerotia g⁻¹ dry shoot tissue reaches rather similar values for inoculum densities of 1, 3, and 10 microsclerotia g⁻¹ soil, *i.e.*, $1.6 \times 10^3 \pm 9.0 \times 10^2$, $2.2 \times 10^3 \pm 6.4 \times 10^2$, and $3.8 \times 10^3 \pm 1.1 \times 10^3$, respectively, if only the stems showing externally visible microsclerotia are taken into account. However, at 30 and especially at

100 microsclerotia g^{-1} soil, higher densities of microsclerotia, viz., $8.5 \times 10^3 \pm 4.3 \times 10^3$ and $2.9 \times 10^4 \pm 6.8 \times 10^3$ respectively were observed in the stems. The much higher density of microsclerotia at 100 microsclerotia g^{-1} soil indicates that repeated infection of the shoot leads to a more thorough colonization and subsequently to higher amounts of microsclerotia g^{-1} shoot. Therefore we hypothesize that a single shoot infection is not able to colonize the shoot completely.

The most sensitive bioassays reported so far recommend the determination of the number of root infections per unit of root length on thorn apple (*Datura stramonium*) (Evans *et al.*, 1974). This method allows a detection limit of about 2-3 microsclerotia g^{-1} soil. The detection limit reported here of at least 1 microsclerotium g^{-1} soil is also lower than with other plants tested such as potato and eggplant (Nagtzaam *et al.*, 1997). This may be due to the high sensitivity of *A. thaliana* to systemic root infection. While in many host plants the great majority of root infections remain localized (Huisman and Gerik, 1989), in *A. thaliana* we observed mainly systemic infections, with microsclerotia present inside the xylem tissue of roots after plating onto Ethanol Agar. It was, therefore, impossible to count the number of root infections in our bioassay. Nevertheless, the percentage of root length occupied by *V. dahliae* 14 days after plating onto MSEA was strongly correlated with soil inoculum density (Figure 2.4). Given the low standard errors, we therefore recommend, in line with Evans *et al.* (1974), to use the amount of root infection as the best estimate for soil inoculum density of *V. dahliae*. If however, the aim is solely to determine whether a given soil is infested with *V. dahliae*, the less labour-intensive method of observing the senescence severity 6-9 weeks after planting 4-week-old seedlings of *A. thaliana* in infested soil may be used.

CHAPTER 3

EFFECT OF TEMPERATURE ON THE FORMATION OF MICROSCLEROTIA OF *VERTICILLIUM DAHLIAE*

Summary

Microsclerotium formation by six isolates of *Verticillium dahliae* was studied at different temperatures both *in vitro* and in *Arabidopsis thaliana*. Mycelial growth appeared to be optimal at 25°C, but microsclerotium formation was greatest at 20°C (two isolates) or 15-20°C (one isolate). Seedlings of *A. thaliana* were root-dipped in a conidial suspension, planted, and either placed at 5, 10, 15, or 25°C, or left at 20°C until the onset of senescence, after which some of the plants were placed at 5, 10, 15, or 25°C. The amount of microsclerotia per unit of shoot weight was assessed in relation to isolate and temperature. Generally, the optimal temperature for production of microsclerotia was 20°C. Two isolates each produced about ten times more microsclerotia than each of the other four isolates. For these isolates, high $R^2_{adj.}$ -values of 0.77 and 0.66 were obtained in multiple regressions, with temperature and its square as highly significant ($P < 0.001$) independent variables. $R^2_{adj.}$ -values for the other isolates varied between 0.28 and 0.39. Moving plants to different temperatures at the onset of senescence led to microsclerotial densities intermediate between densities on plants that had grown at constantly 20°C and plants grown at other temperatures. This suggests that vascular colonization rate and rate of microsclerotium formation are similarly affected by temperature. The senescence rate of plants appeared unimportant except for plants grown at 25°C, which showed highest amounts of microsclerotia per unit of plant weight in the most rapidly senescing plants. Temperature and isolate are regarded as primary factors in the microsclerotium formation in host tissue.

Introduction

Verticillium dahliae Kleb. is a soil-borne wilt pathogen of a wide range of crops such as vegetables (*e.g.* potato, tomato, eggplant), ornamentals (*e.g.* chrysanthemum), fruit trees (*e.g.*, cacao, olive), and shade trees (*e.g.* ash, maple) (Pegg, 1974; Schnathorst, 1981; Hiemstra, 1998). The pathogen can survive for 14 years or more (Wilhelm, 1955) in soil as microsclerotia, which

are small, multi-celled and melanized structures. Germination of microsclerotia in the rhizosphere is induced by root exudates (Schreiber and Green, 1963; Schnathorst, 1981). After penetration of the vascular system, the pathogen is transported passively upwards through the xylem vessels. Disease symptoms develop as the result of physical blockage of the xylem vessels and the production of toxins. Finally, verticillium wilt results in early senescence of the infected plant. When infected plant parts are dying or dead, the pathogen grows into the parenchymatous tissue where it forms abundant microsclerotia up to $2 \times 10^5 \text{ g}^{-1}$ dry shoot tissue or more (Mol and Scholte, 1995a). However, among and within fields the formation of microsclerotia in shoot tissue can vary considerably (Mol and Scholte, 1995b). Although it seems likely that spatial pattern in inoculum density explains most of the spatial variation in formation of microsclerotia (Smith and Rowe, 1984), it may at the same time be the consequence of variation in formation of microsclerotia. The choice of host cultivar has been shown to strongly influence density of microsclerotia in host tissue (Slattery, 1981; Davis *et al.*, 1983). In addition, environmental factors may play an important role.

Temperature may affect formation of microsclerotia either directly or indirectly through plant senescence. Heale and Isaac (1965) reported that the optimum temperature for microsclerotium formation by *V. dahliae* produced on Czapek-Dox Agar was 24°C. Brinkerhoff (1969) reported on PDA optimum temperatures for mycelial growth and formation of microsclerotia of 22-25°C. In cotton leaves, some formation of microsclerotia continued even at 5 and 30°C, though with low densities, but it was stopped at 32°C (Brinkerhoff, 1969). Formation of microsclerotia in infected tomato tissue in unsterile soil amended with Potato-Dextrose Broth occurred at all temperatures tested (12-33°C) and was optimal at 24°C (Ioannou, 1977a).

The goal of the present study was to investigate the effect of temperature on microsclerotium formation *in vitro* and *in planta*. *Arabidopsis thaliana* was used as a test plant because of its high susceptibility to *V. dahliae* and short life cycle (Chapter 2). To avoid variation due to differences in inoculum density, seedlings were root-dipped in a high density conidial suspension. To study temperature effects on events prior to microsclerotium formation, plants grown at constant temperatures were compared to plants left at 20°C until the first symptoms of senescence were observed, after which they were placed at the test temperatures of 5, 10, 15, and 25°C.

Materials and methods

Inoculum of V. dahliae

Six monospore cultures of *V. dahliae* were obtained from a flax stem (isolate C1) or potato stems (isolates C2, C3, V1, V3, V4) collected from fields in the province of Gelderland (isolates C1-C3) or Drenthe (isolates V1-V4), the Netherlands. Conidial suspensions were prepared by growing the cultures in Czapek-Dox liquid medium (Oxoid) for 7 days at 23°C in a shaking incubator. After incubation, conidia were filtered through cheese cloth and suspended in sterile distilled water to obtain 10^6 conidia ml⁻¹.

Effect of temperature in vitro

Cultures of isolates V1-V4 were grown on Ethanol Agar medium (Nadakavukaren and Horner, 1959) at 10, 15, 20, 25, or 30°C in the dark for 25 days. Colony size was measured every 2-3 days and numbers of microsclerotia per colony were counted at the end of the incubation time. To determine the numbers of microsclerotia, a measured sector of the colony was taken, ground using mortar and pestle, suspended in 0.08% water agar, and the microsclerotia were directly counted in three small subsamples (each 15 µl) of the suspension using a dissecting microscope (magnification 25 ×).

Effect of temperature in planta

Seedlings of *Arabidopsis thaliana* ecotype Columbia were germinated in steamed potting soil. After 40 days at 22°C the first rosette leaves were formed and the plants uprooted. The roots were washed in sterile distilled water, dipped for 20 min in a conidial suspension, planted in black plastic pots (w/w/h, 7/7/6 cm) containing sieved potting soil mixed with sand (2/1, v/v), and placed at 5, 10, 15, 20, or 25°C for experiment 1 (isolates C1-C3) or 10, 15, 20, or 25°C for experiment 2 (isolates V1-V4) respectively. Another set of plants was placed first at 20°C and at the onset of senescence transferred to the temperatures of 5 (experiment 1 only), 10, 15, or 25°C. The onset of senescence was defined as the stage when rosette leaves and lower stem leaves had started to yellow and first seeds were being produced. At this stage, no microsclerotia could be observed within the plant tissue. Every treatment was replicated ten (experiment 1) or six (experiment 2) times. Plants were watered as necessary and placed in a 16 h-lighted (Philips TD 32 W/84 HF) climate chamber.

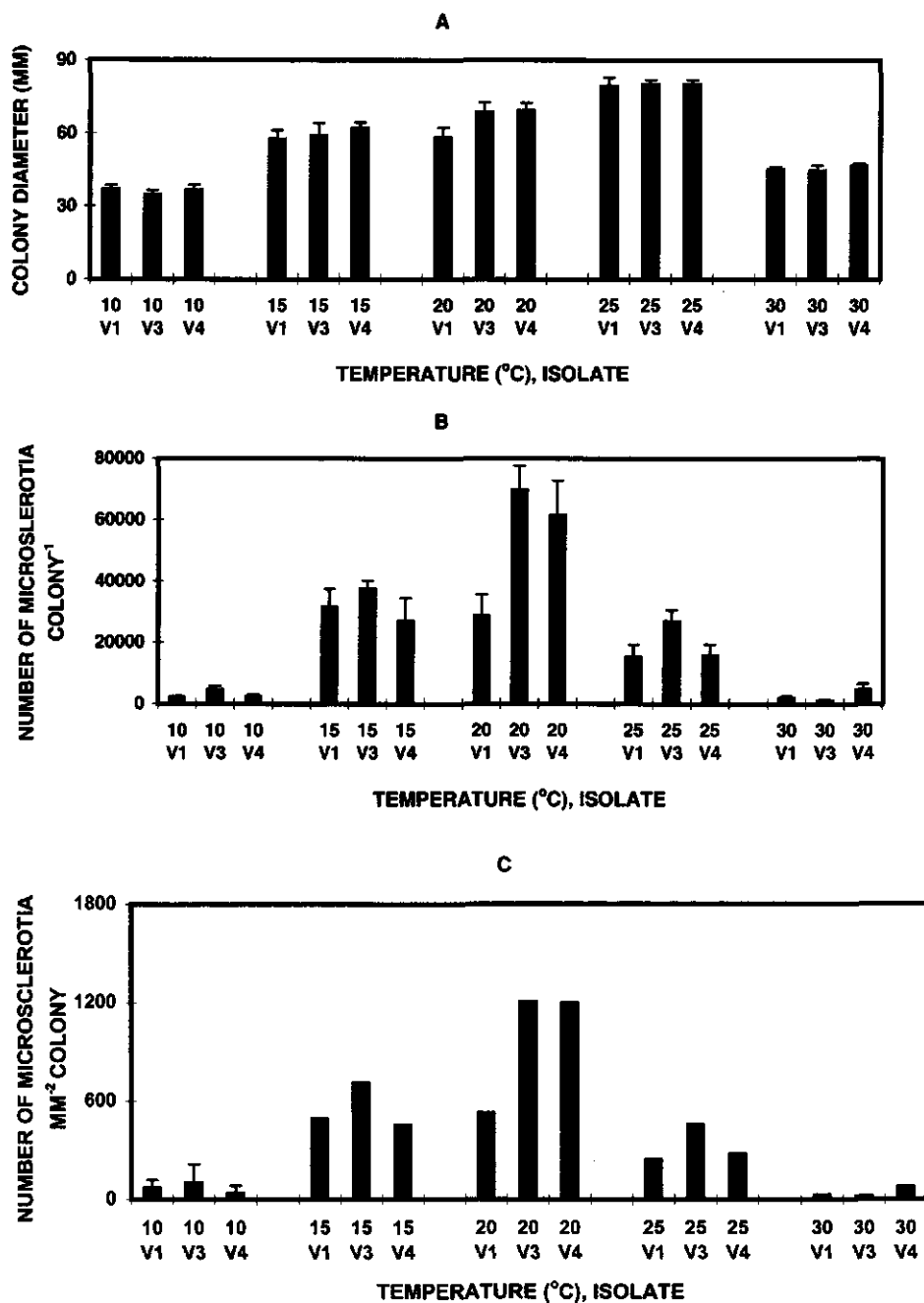


Figure 3.1. Effect of temperature on three isolates of *Verticillium dahliae* growing on Ethanol Agar. (A) Colony diameter, (B) Number of microsclerotia per colony, (C) Number of microsclerotia mm⁻² colony.

Plants were harvested after when dead. After cutting off the shoot, pots were soaked in standing tap water for 30 min and the soil was removed from the roots. Shoots and roots were dried, weighed, and stored separately in paper bags until further analysis. Both dried shoots and roots were ground separately using a mortar and pestle and suspended in 0.08% water agar. The density of microsclerotia in the suspension was determined by direct counting of at least three droplets of known volume (approximately 20 μ l) under a dissecting microscope.

Numbers of microsclerotia were transformed logarithmically ($\log_{10}(x+1)$) prior to data analysis. The experimental design was a split-plot design with temperature as main-plot factor and constant versus different temperature and *V. dahliae* isolate as sub-plot factors.

Results

Effect of temperature on mycelial growth and microsclerotium formation in vitro

Maximum growth occurred at 25°C for all isolates, with growth at 15 and 20°C only slightly less (Figure 3.1A). Growth at 10 and 30°C was about half the growth at 25°C ($P < 0.001$). Growth rate of three isolates (C1 - C3) was broadly similar. Formation of microsclerotia per colony and per mm² colony varied considerably ($P < 0.001$), with an obvious maximum at 20°C for isolates V3 and V4, and a maximum at 15-20°C for isolate V1 (Figure 3.1B,C).

Effects of V. dahliae and temperature on Arabidopsis thaliana

The effect of *V. dahliae* on growth of *A. thaliana* was determined only in experiment 2. Plants grown at lower temperatures generally grew more slowly and yielded higher dry weights. On average, uninoculated plants grown continuously at 10, 15, 20, and 25°C were completely dead 293, 246, 178, and 99 days after inoculation respectively (Figure 3.2). Inoculation with *V. dahliae* accelerated senescence by 26, 36, 45, and 34% respectively, as compared to the uninoculated plants (Figure 3.2). Inoculated plants weighed 7-67% less than control plants (Figure 3.3). The strongest growth reduction occurred for all three isolates at 25°C. The negative effect of isolate V1 on plant dry weight was more apparent at higher temperatures: a 9% reduction at 5°C up to 67% at 25°C. For isolates V3 and V4, growth reduction was also considerable at 10 and 15°C, with a range of 20-28% and 21-25% respectively. It is remarkable that the growth reduction observed with isolate V4 at 20°C was only 7%. Root dry weights were quite low and highly variable, probably because they had already been partly decomposed. Shoot dry weight and life span of individual plants were significantly ($P < 0.001$) and positively correlated (Pearson $R =$

0.72). However, the reduction of shoot dry weight due to *V. dahliae* was not correlated with the reduction of life span due to *V. dahliae*.

Plants grown at 20°C and moved to 10, 15, or 25°C at the onset of senescence showed more similar life spans and dry weights to those of plants grown continuously at 20°C than those of plants grown continuously at 10, 15, or 25°C (Figures 3.2, 3.3). Thus, plants moved to 10 or 15°C had shorter life spans, and those moved to 25°C had longer life spans than plants that had been grown at these temperatures continuously. Except for isolate V1 at 25°C, all plants that had been moved had greater reductions in dry weight and life spans due to *V. dahliae* than plants that grew continuously at the same temperature (Table 3.1).

Analysis of variance indicated a highly significant interaction ($P < 0.001$) between isolate, temperature, and the act of moving plants to different temperatures at the onset of senescence, on the density of microsclerotia in shoot tissue. The average number of microsclerotia formed per gramme dry weight was almost identical for shoot and root, 1.4×10^5 and 1.3×10^5 respectively (averages from untransformed data). Isolates C1, V1, V3, and V4 formed on average 8.8×10^3 – 1.8×10^4 microsclerotia g^{-1} shoot dry weight, whilst isolates C2 and C3 formed on average $2.9 \times$

Table 3.1. Average percentage of reduction of shoot dry weight and life span of the test plants due to *Verticillium dahliae*. Constant = plants grown continuously at temperatures indicated; Changed = plants grown at 20°C and moved to the temperatures indicated at the onset of senescence.

Isolate	Temperature (°C)	Temperature treatment			
		Constant	Changed	Constant	Changed
		Plant dry weight		Life span	
V1	10	9.4	51	19	21
	15	10	58	18	45
	20	38	-	39	-
	25	67	47	31	31
V3	10	20	25	13	33
	15	21	35	21	48
	20	11	-	43	-
	25	30	53	30	35
V4	10	28	45	19	21
	15	25	41	21	46
	20	6.8	-	39	-
	25	66	76	21	26

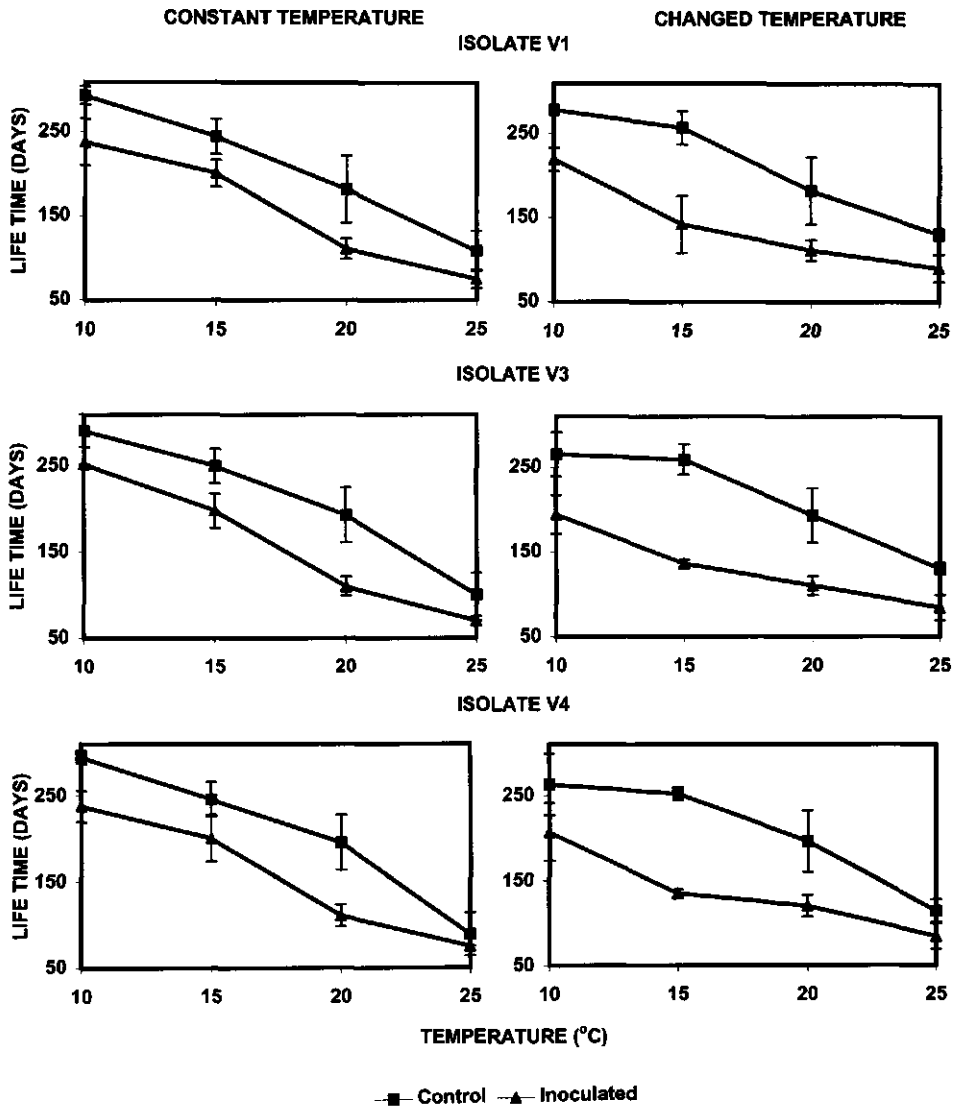


Figure 3.2. Effect of three isolates of *Verticillium dahliae* and of temperature on life span of *Arabidopsis thaliana*. Plants were maintained either at constant temperatures, or they were grown up at 20°C and placed at the indicated temperatures at the onset of senescence ('changed temperature').

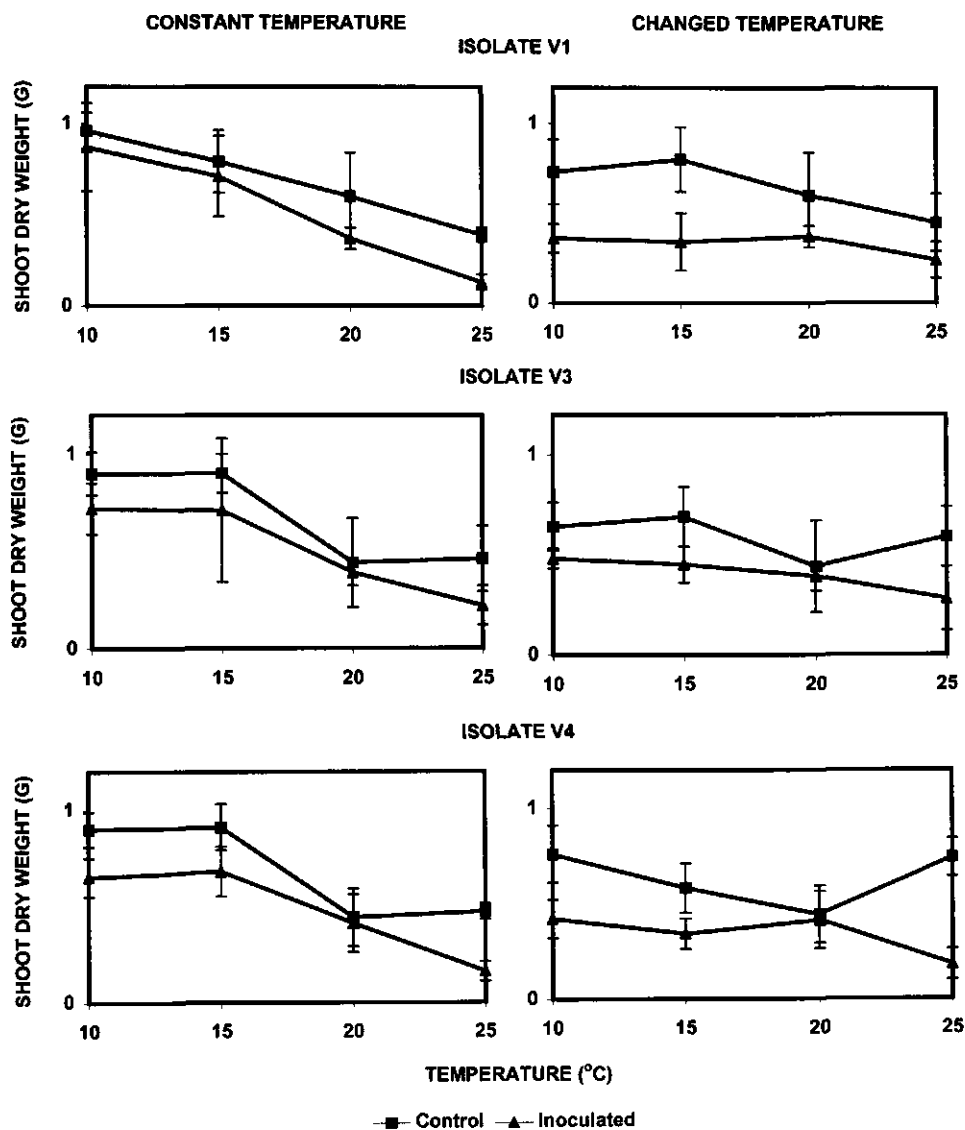


Figure 3.3. Effect of three isolates of *Verticillium dahliae* and of temperature on shoot dry weight of *Arabidopsis thaliana*. Plants were maintained either at constant temperatures, or they were grown up at 20°C and placed at the indicated temperatures at the onset of senescence ('changed temperature').

$10^5 - 3.0 \times 10^5$ microsclerotia g^{-1} shoot dry weight (averages from untransformed data). Most isolates showed an optimum microsclerotium production per unit shoot dry weight at 15-20°C (Table 3.2). Isolates V3 and V4 showed almost identical production of microsclerotia at 15 and 20°C.

The numbers of microsclerotia g^{-1} shoot dry weight for plants grown at constant temperatures and for plants moved from 20°C to different temperatures at the onset senescence was 1.6×10^5 and 1.5×10^6 (averages from untransformed data) respectively. The effect of moving plants to different temperatures at the onset of senescence generally led to microsclerotial densities in plant tissue closer to those of plants grown at constant 20°C. In most cases the optimum temperature for microsclerotium formation of moved plants remained 15-20°C. For isolates C1 and C3, the effect of moving plants at the onset of senescence to 5°C resulted in a significant ($P < 0.01$) increase in the density of microsclerotia g^{-1} shoot dry weight and of number microsclerotia per plant (data not shown) compared to plants that had grown continuously at 5°C (Table 3.2). For isolates C1, C2, C3, and V1, a similarly significant effect was found for plants that had been moved to 10°C. However, the effect of moving plants to different temperatures was largely insignificant for isolates V3 and V4. Movement of plants to 25°C led to a significant increase in the number of microsclerotia per shoot dry weight for two isolates and a significant decrease for one isolate. Similar effects were found for the production of microsclerotia on the root, although these optima were less prominent for isolates C2 and C3 (data not shown). Attempts to describe the density of microsclerotia in plant material for all isolates and treatments in one (multiple) regression as a function of temperature, the square of the temperature, plant, shoot, or root dry weight, and/or pathogenicity of the isolate (defined as the effect of the isolate on growth reduction) using multiple regression led to a maximum $R^2_{adj.}$ of 0.28 with the square of the temperature and dry shoot and root weight as significant terms ($P < 0.01$). When only the separate isolates treated at constant temperatures were considered, high $R^2_{adj.}$ values of 0.77 and 0.66 were obtained only for isolates C2 and C3, with temperature and its square as highly significant ($P < 0.001$) terms. $R^2_{adj.}$ values for the other isolates varied between 0.28 and 0.39, with the temperature and its square (isolate C1) or effect of the isolate on growth reduction (isolates V1-V4) as significant ($P < 0.01$) terms.

No significant correlations between life span and density of microsclerotia in host tissue were found when all the data were considered. However, significant correlations between life span and density of microsclerotia in host tissue were found for plants grown at 25°C, where a significant negative correlation was found (Pearson $R = -0.62$). Plants at this temperature containing the highest microsclerotia densities had the shortest life spans.

Discussion

The temperature optimum for formation of microsclerotia *in vitro* (15-20°C) is lower than that for hyphal growth of *V. dahliae* (25°C). With *A. thaliana*, an optimum of 20°C was found for the formation of microsclerotia by most isolates. We were unable to describe microsclerotial density as a function of other variables measured, such as plant dry weight, amount of microsclerotia in the root, or the effect of *V. dahliae* on plant, shoot or root dry weight. Isolates C2 and C3 produced about ten times more microsclerotia than the other isolates. When the individual isolates were considered, regression resulted in high R^2_{adj} -values for isolates C2 and C3 only, where temperature and its square were highly significant. Isolates C2 and C3 produced *c.* ten times more microsclerotia per unit plant weight than the other isolates. Microsclerotium production in these other isolates (C1, V1, V3, V4) may be limited by factors that are different to those in isolates C2 and C3.

Mol *et al.* (1996b) observed that soil inoculum density of *V. dahliae* after incorporation of debris of a range of hosts and non-hosts interacted strongly with the *V. dahliae* isolate used as inoculum. An isolate of *V. dahliae* obtained from a soil cropped continuously to potato for 14 years led to higher inoculum densities when potato was grown than when beans were grown; and an isolate obtained from a soil cropped continuously to beans for 10 years led to higher inoculum densities when beans were grown than when potato was grown (Mol *et al.*, 1996a). We found that different isolates originating from potato (except isolate C1 from flax), can lead to widely different numbers of microsclerotia in host tissue. It can be expected that in the field these isolates would quickly outcompete the other isolates. However, other conditions, such as soil moisture and its persistence may also influence the rate of success of particular isolates.

Soesanto and Termorshuizen (Chapter 2) found that the density of microsclerotia formed in shoot tissue depends on the soil inoculum density, and concluded that multiple root and vascular infections are needed to obtain maximum quantities of microsclerotia in the shoot. In the present experiments, a high level of root infections was ensured by applying a root dip inoculum of 10^6 conidia ml⁻¹, and consequently the number of root infections was not a limiting factor. An effect of temperature on vascular colonization rate may explain the difference in microsclerotial densities between moved and unmoved plants at low temperatures. It is probable that *V. dahliae* grew and colonized more tissue at 20°C than at 5 or 10°C. The higher biomass of *V. dahliae* in the vascular system resulted in more microsclerotia produced for the moved plants. When plants were moved to different temperatures, the formation of microsclerotia had yet to begin; consequently the fewer microsclerotia produced in plants moved to low temperatures compared

Table 3.2. Effect of six isolates of *Verticillium dahliae* and temperature on production of microsclerotia in senescent tissue of *Arabidopsis thaliana*.

Temperature (°C)	Isolate											
	C1		C2		C3		V1		V3		V4	
	Constant ¹	Changed ²	Constant	Changed	Constant	Changed	Constant	Changed	Constant	Changed	Constant	Changed
5	3.6×10 ³ a ^{3,4,5}	1.1×10 ⁴ ab*	1.6×10 ⁴ a	3.4×10 ⁴ a	6.1×10 ³ a*	3.7×10 ⁴ a*	-	-	-	-	-	-
10	7.5×10 ³ a*	2.7×10 ⁴ c*	9.5×10 ⁴ b*	3.1×10 ⁵ c*	8.1×10 ⁴ bc*	5.3×10 ⁵ b*	2.7×10 ³ a*	6.4×10 ³ a*	4.6×10 ³ a	4.8×10 ³ a	6.2×10 ³ a	5.0×10 ³ a
15	2.8×10 ⁴ b*	1.1×10 ⁴ ab*	5.3×10 ⁵ c*	3.7×10 ⁴ ab*	5.0×10 ⁴ c	2.4×10 ⁴ a	4.8×10 ³ a	7.5×10 ³ a	9.0×10 ³ a	6.2×10 ³ a	1.3×10 ⁴ a	6.1×10 ³ a
20	7.9×10 ³ a	7.9×10 ³ b	5.2×10 ⁵ c	5.2×10 ⁵ c	3.2×10 ⁵ d	3.2×10 ⁵ b	1.2×10 ⁴ a	1.2×10 ⁴ a	1.2×10 ⁴ a	1.2×10 ⁴ a	1.2×10 ⁴ ab	1.2×10 ⁴ a
25	5.3×10 ³ a	1.9×10 ⁴ bc	2.4×10 ⁵ c*	7.6×10 ⁴ b*	2.3×10 ⁵ cd*	3.4×10 ⁴ a*	1.6×10 ³ a	1.8×10 ⁴ a	1.1×10 ⁴ a	1.0×10 ⁴ a	2.7×10 ³ b*	8.9×10 ³ a*
Isolate average	1.0×10 ⁴ a ⁶		1.3×10 ⁵ b		7.9×10 ⁴ b		6.3×10 ³ a		8.3×10 ³ a		7.3×10 ³ a	

¹ Plants grown continuously at temperatures indicated.

² Plants grown at 20°C and moved to temperatures indicated at the onset of senescence.

³ Values are back-transformed from log₁₀(x+1)-transformed data, where x = number of microsclerotia g⁻¹ shoot dry weight.

⁴ Values followed by the same letter in a column do not differ significantly, according to Tukey's HSD test (*P* < 0.05).

⁵ Values followed by an asterisk indicate a significant difference between 'constant' and 'changed' according to Tukey's HSD test (*P* < 0.05).

⁶ Isolate average values followed by the same letter do not differ significantly, according to Tukey's HSD test (*P* < 0.05).

to those grown at constant 20°C may be a direct effect of temperature on the rate of formation of microsclerotia.

In addition to shoot colonization rate and rate of formation of microsclerotia, the rate of senescence of plant material may also determine the final density of microsclerotia in host plant tissue. However, no significant correlations between life span and density of microsclerotia in host tissue were found. On the one hand, at low temperatures, senescence progressed slowly giving ample time for development of microsclerotia. On the other hand, at suboptimal temperatures *V. dahliae* may become outcompeted by other organisms colonizing senescent or dead host tissue. These two processes may counterbalance each other. Significant correlations between life span and density of microsclerotia in host tissue were not found if the temperature treatments were analysed separately, except at 25°C, where a significant negative correlation was found. Plants at this temperature containing the highest densities of microsclerotia had the shortest life spans, and consequently the fastest rates of senescence. This indicates that a slow rate of senescence is not particularly advantageous to *V. dahliae*. When senescence is too slow, *V. dahliae* might be partially outcompeted by saprotrophic organisms.

Root weight was at least 10 times less than shoot weight. We suppose that this is due to the decomposition of part of some root material and therefore root measurements are not reliable enough to warrant detailed discussion. The high microsclerotial densities in the root tissue are likely to be a consequence of the root dip inoculation method. Often microsclerotial densities have been reported to be higher in shoot than in root tissue (Lacy and Horner, 1966), although Mol and Scholte (1995a) reported that densities of microsclerotia g⁻¹ roots or stolons of potato were about one quarter of those on stems.

Microsclerotia are the survival structures of *V. dahliae* that compose the inoculum for subsequent crops. Knowledge about environmental factors affecting their formation could contribute to new methods of managing verticillium wilt. However, to understand the dynamics of *V. dahliae* during the colonization of senescence plant material more detailed studies on the induction of microsclerotium formation and on the effect of competition during the saprotrophic stage of *V. dahliae* are needed.

CHAPTER 4

RECOVERY OF MICROSCLEROTIA OF *VERTICILLIUM DAHLIAE* FROM SOIL AS SUBJECTED TO VARIOUS TREATMENTS

Summary

The influence of various conditions on the survival of microsclerotia of *Verticillium dahliae* was studied using three sources of field-collected potato stems densely covered with naturally-formed microsclerotia. Microsclerotia were found to survive for up to two years in potato stems not incorporated in soil, while under various conditions of temperature and relative humidity. The effects of temperature, pF, including weekly variations in temperature and/or pF, and various modes of incorporating potato stem tissue on the survival of microsclerotia for up to one year in a sandy unsterilised soil was also studied for different microsclerotia sources. Inoculum source had no significant effect. Remarkably few microsclerotia were recovered one day after the start of experiments varying between 5.5 and 31%. Recovery remained at this level or even decreased for another month and, for several treatments, also after 3 and 6 months. Only after 3 to 12 months, recovery increased to values up to 5 times higher than that of one day after start of the experiment, but recoveries did not exceed the number of microsclerotia initially incorporated into the soil. Changes in recovery may be due to variation in the level of soil mycostasis which is affected by the rate of nutrient exudation from microsclerotia.

Introduction

Verticillium dahliae Kleb. is the causal agent of wilt disease in many crops, including potato, cotton, olive, and strawberry (Schnathorst, 1981). In the absence of host plants the fungus can survive in soil for many years as microsclerotia (Wilhelm, 1955), which are formed predominantly in the senescent host tissue. Crop rotation using non-hosts is an important management strategy to reduce yield losses (Bollen *et al.*, 1989).

Data on the long-term persistence of microsclerotia of *V. dahliae* in field soils are scarce. Wilhelm (1955) reported that *V. dahliae* may survive in field soils for at least 14 years. Long-term observations on the persistence of *V. dahliae* in the field are, however, confounded by production of new microsclerotia on non-hosts (Malik and Milton, 1980). Probably only a minor fraction of microsclerotia remains viable during several years since yield losses in susceptible potato cultivars are nullified in a crop rotation with 3 years of non-hosts (Bollen *et al.*, 1989). Slattery (1981) studying the survival of microsclerotia of *V. dahliae* in potato stems buried in field soil during winter for 7 months found survival rates as low as 23%. In other studies on the survival of microsclerotia, total mortality was found after 5 years (Green, 1980). Green (1980) concluded that temperature and soil moisture had less influence than soil type on survival of microsclerotia. Hawke and Lazarovits (1994) found that survival of *in vitro* produced microsclerotia less than 53-75 µm diam. stored without soil declined quickly after 16 weeks incubation, while those measuring 75-106 µm diam. survived fully for 36 weeks. Mortality of these larger microsclerotia buried in soil depended on soil type and varied between 0 and 24% after 4 weeks of exposure.

In this paper we report on the recovery of microsclerotia of *V. dahliae* as a function of temperature, moisture, and the incorporation of organic debris. Our hypothesis was that fluctuating temperature and/or pF would cause an initially rapid decrease in survival.

Materials and methods

Incubation of microsclerotia without soil: effect of temperature and relative humidity (Experiment 1)

Inoculum of *Verticillium dahliae* consisted of naturally formed microsclerotia on dead potato stems collected from three potato fields in the Province of Drenthe, the Netherlands, in October 1996. The three sources of inocula were designated as V1, V3 and V4. After collection, the stems were cut into 10-cm-long pieces, allowed to air-dry for about 10 days under ambient conditions at room temperature, and subsequently stored in plastic bags until further use. For each inoculum source, 30 pieces of dried 10-cm-long potato stems covered with microsclerotia were placed in a plastic box (l/w/h, 25/10/9 cm) on a plastic screen table (height 6 cm) and loosely covered with a lid. Stem pieces were incubated at relative air humidity of 100 or 70%. To establish a relative air humidity of 100%, a 3-cm-layer of water was added into the plastic box. A saturated NaCl solution was added to maintain a relative air humidity of 70% (Winston and Bates, 1960). Boxes

were incubated in a climate chamber at 5 or 20°C for 0, 3, 6, 12, or 24 months. Each temperature × relative humidity combination was replicated three times.

At each sampling occasion, three potato stems were taken randomly from each box, ground using a mortar and pestle, and sieved using meshes of 106 and 20 µm. Material remaining on the 20 µm sieve was placed on filter paper under a dissecting microscope and 75 microsclerotia were picked and plated onto Modified Soil Extract Agar (MSEA, Harris *et al.*, 1993) amended with 50 ppm oxytetracyclin using an insect pin 000 (Emil Arlt, Australia). Each plate contained 25 microsclerotia. Plates were placed upside down and incubated in the dark at 23°C for three weeks. The numbers of colonies of *V. dahliae* were subsequently counted. The germination percentages of the three inoculum sources plated three weeks prior to the start of the experiment were 67, 67, and 89% for V1, V3, and V4 respectively.

Incubation of microsclerotia in soil (Experiments 2-4)

Inoculum of *Verticillium dahliae* in dead potato stems collected from three different potato fields in the Province of Drenthe, the Netherlands, in October 1997 and were designated K1, K2, and K3. After collection, the stems were cut into 10-cm-long pieces, allowed to air-dry for about 10 days at room temperature, and subsequently stored in plastic bags until further use. Germinability determined 3-5 weeks prior to the start of the experiment was 94-95%.

A sandy agricultural soil from Meterik (Province of Limburg, the Netherlands) was checked for presence of microsclerotia of *V. dahliae* largely following the method described by Harris *et al.* (1993). Twelve and a half grams of soil was air-dried for 14 days, and sieved over 106 and 20-µm screens. The material remaining on the 20-µm sieve was suspended in 50 ml 0.08% water agar and 10 0.8 ml-subsamples from the soil suspension were plated onto 10 Petri dishes containing MSEA. Plates were incubated in the dark at 23°C for three weeks and subsequently the number of colonies of *V. dahliae* was counted.

For experiments 2 and 3, microsclerotial suspensions were prepared by grinding dried inoculum in water using mortar and pestle, sieving over 106 and 20-µm screens, and suspending the material remaining on the lower screen in a small, measured amount of water. The numbers of microsclerotia ml⁻¹ suspension were directly counted in small subsamples under the dissecting microscope. The suspension was diluted to achieve numbers of microsclerotia in an amount of water that would lead, after thoroughly hand-mixing with the soil, to 100 microsclerotia g⁻¹ air-dry soil and a pH of 2.0 or 2.8. Small plastic pots (h/diam., 5/3 cm) were filled with 75 g of the infested soil, and incubated under different conditions as described below. The pots had lids with a small opening to allow for oxygen exchange. The pots were kept at constant weight by hand

watering. Samples were taken after 0, 1, 3, 6, or 12 months. The first sample ($t = 0$) was taken 1 day after incorporation of the microsclerotia into the soil. At each sampling occasion, the whole 75 g of soil was used, so for every sample different pots were used. The density of microsclerotia in soil was determined as described above (Harris *et al.*, 1993). All experiments were carried out in a completely randomized block design with 3 blocks.

Effect of temperature (Experiment 2)

Small plastic pots were filled with soil containing 100 microsclerotia g^{-1} air-dry soil of inoculum sources K1, K2, or K3 at a pF of 2.0. Treatments included constant temperature (-28 , 5 , or 25°C), or weekly varying temperature ($-28/+5^\circ\text{C}$; or $-28/+25^\circ\text{C}$).

Effect of temperature and moisture content (Experiment 3)

Small plastic pots were filled with soil containing 100 microsclerotia g^{-1} air-dry soil of inoculum K3 at pF of 2.8. Additional water was added to part of the pots to reach a pF of 2.0. Treatments

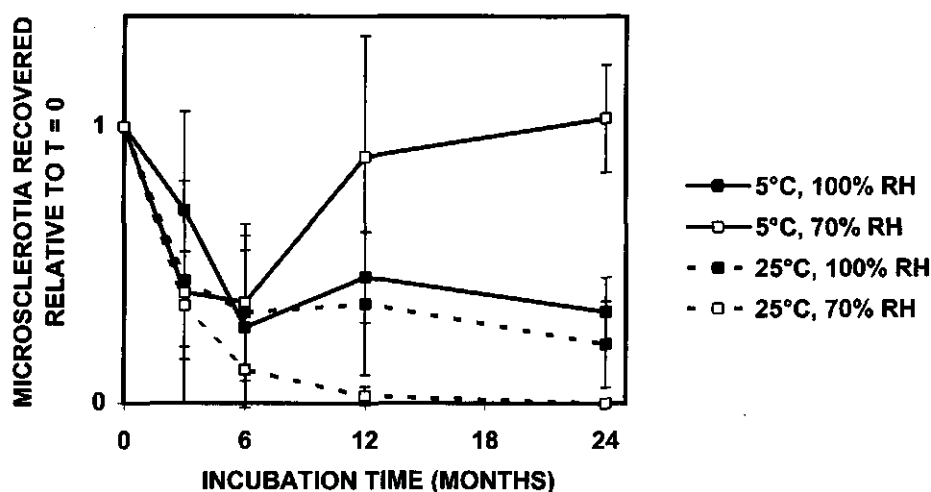


Figure 4.1. Effect of temperature, relative humidity, and time on the recovery of microsclerotia of *Verticillium dahliae* in field-collected 10-cm-long, dead potato stems incubated on plastic screens. The recovery is expressed as the proportion of germinating microsclerotia of *Verticillium dahliae* relative to the germination at $t = 0$. Germination percentage was determined by plating 75 single microsclerotia to Modified Soil Extract Agar plates. The experiment included three different sources of inoculum. Since no differences were observed among inocula, data were merged. Error bars indicate standard deviations of the means for the three inocula. At $t = 0$, average germination percentage was 74%.

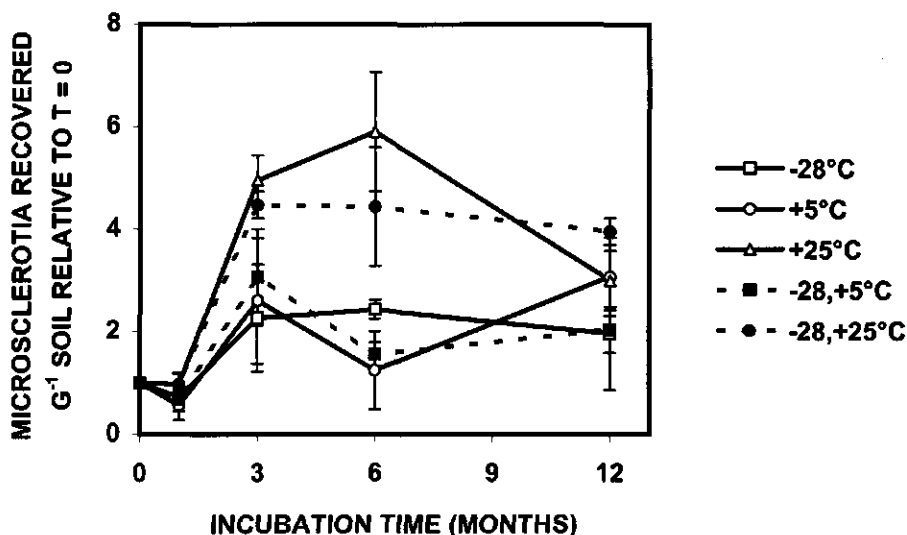


Figure 4.2. Effect of constant temperatures (-28 , $+5$, or $+25^{\circ}\text{C}$) or weekly changing temperatures ($-28/+5^{\circ}\text{C}$ or $-28/+25^{\circ}\text{C}$) on the recovery of microsclerotia of *Verticillium dahliae* in an unsterilized sandy soil. The number of microsclerotia g^{-1} air-dry soil relative to the number at $t = 0$ is shown. At $t = 0$, 100 germinable microsclerotia g^{-1} air-dry soil were incorporated. Prior to incubation, germination percentage as determined by plating single microsclerotia was 94–95%. The experiment included three different inoculum sources. Since no differences were observed among inocula, data were merged. Average recovery of microsclerotia one day after incorporation ($= t = 0$) was 13% (see text). Error bars indicate standard deviations of the means of the three inocula.

included temperature (constant 5, 15, or 25°C , or weekly alternating $5/25^{\circ}\text{C}$) and soil moisture (constant pF 2.0 or 2.8, or monthly alternating pF 2.0/2.8) regimes in all possible combinations, giving a total of 12 different treatments. The pots treated with alternating $5/25^{\circ}\text{C}$ and/or pF 2.0/2.8 started at 5°C and pF of 2.8.

Effects of adding plant tissue (Experiment 4)

The three *V. dahliae* inocula K1, K2, and K3 were incorporated in four ways: (1) 3 g of 1-cm-long pieces of potato stems containing microsclerotia were buried in the soil, (2) the material as in (1) was ground using mortar and pestle, (3) as (2) but the ground material was sieved over 106 and 20- μm screens, and the material remaining on the upper sieve was autoclaved (121°C for 20 min) before incorporation in the soil, and (4) as (2) but without incorporation of the potato tissue

remaining on the 106 μm sieve in soil. Stem pieces in treatment 1 were incorporated after soil had been moistened to pF 2.0 by careful hand-mixing. Ground materials (treatments 2-4) were incorporated into soil by suspending in an amount of water that after thorough hand-mixing resulted in a pF of 2.0. The density of microsclerotia in soil was determined as described above (Harris *et al.*, 1993). The remaining stem segments were ground in a mortar, sieved on meshes of 106 and 20 μm , suspended in 50 ml 0.08% water agar and 10 0.8 ml-subsamples from the suspension were plated onto 10 Petri dishes containing MSEA. Plates were incubated in the dark at 23°C for three weeks and subsequently the number of colonies of *V. dahliae* was counted and merged with those from soil in the same treatment.

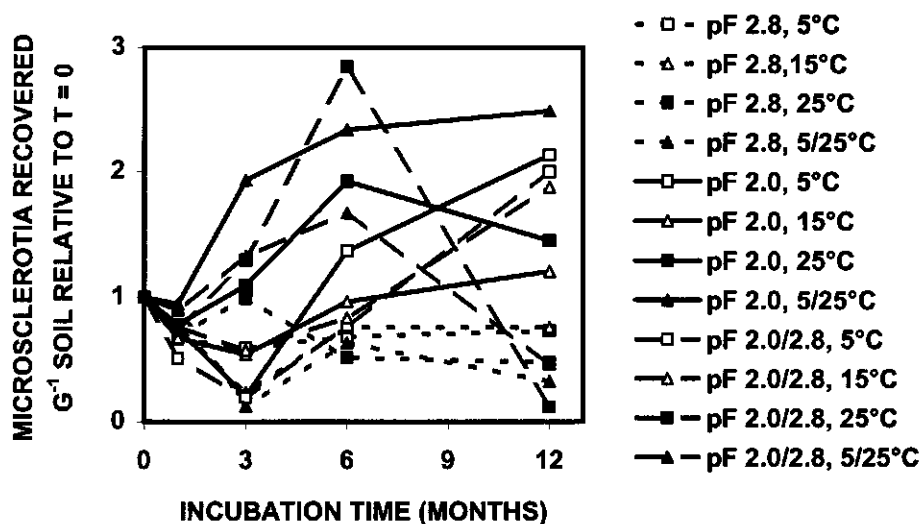


Figure 4.3. Effect of constant or weekly changing temperature (5, 15, 25, or 5/25°C) and pF (2.0, 2.8, or 2.0/2.8) on the recovery of microsclerotia of *Verticillium dahliae* in an unsterilised sandy soil. The number of microsclerotia g^{-1} air-dry soil relative to the number at $t = 0$ is shown. At $t = 0$, 100 microsclerotia g^{-1} air-dry soil were incorporated. Prior to incubation, germination percentage as determined by plating single microsclerotia was 94–95%. The experiment included only one source of inoculum. Average recovery of microsclerotia one day after incorporation ($t = 0$) in soil was 7.7–22% (see text).

Results

The effects of temperature and relative humidity on the germination percentage of microsclerotia from potato stems placed on a plastic screen (experiment 1) did not differ significantly for the three inoculum sources. Therefore, the data for the three sources were merged. The germination percentage of microsclerotia gradually decreased for all treatments with exception of incubation at 5°C and 70% relative humidity (Figure 4.1) which increased to levels equal to those at the onset of the experiment. No microsclerotia were observed only for the 25°C, 70% relative humidity treatment.

The three inoculum sources also gave similar results in response to incubation in soil at varying temperatures (experiment 2), and these data were combined. To illustrate this, at the first sampling occasion one day after start of the experiment ($t = 0$), density of microsclerotia recovered from the soil was 14, 14, and 12% relative to the 100 germinable microsclerotia added to the soil for inocula K1, K2, and K3 respectively. After one month, densities recovered were 7.3 – 12% (Figure 4.2). However, later harvests consistently yielded higher recoveries, varying between 1.2 and 5.9 times more than the level observed at the start of the experiment. After 12 months of incubation, no significant differences were found between treatments. The maximum recovery of microsclerotia found was $81 \pm 14\%$ of the added microsclerotia for inoculum K1 after 6 months of incubation at 25°C.

In experiment 3, the density of microsclerotia recovered directly after the start varied between 5.5 and 31%. The density of microsclerotia recovered from soil varied markedly in time and for the different treatments (Figure 4.3). For all treatments the density recovered decreased after 1 month. For some treatments the recovery then increased, whereas for others it further decreased. The maximum and minimum densities recorded were 44 and 1.1% respectively. Even after initially low observed densities, considerable increases were recorded at subsequent sampling occasions. For example, percentages of microsclerotia recovered at incubation of pF 2.0 and 5°C were found to be $2.9 \pm 1.7\%$ after 3 months, and after 6 and 12 months, they were 21 ± 11 and $32 \pm 9.4\%$ respectively. No obvious pattern was found. Only in the dryer soil (pF 2.8) densities sometimes exceeded those originally introduced.

In experiment 4, when whole stems were incubated, densities recovered after 1 day incubation were 3.7×10^3 , 4.7×10^3 , and 4.2×10^3 microsclerotia g^{-1} soil for inoculum sources K1, K2, and K3 respectively. With the other treatments some losses may have occurred during handling of the potato stems and consequently densities recovered after 1 day incubation were lower: on average 1.5×10^3 , 1.2×10^3 , and 2.7×10^3 microsclerotia g^{-1} soil for inoculum sources

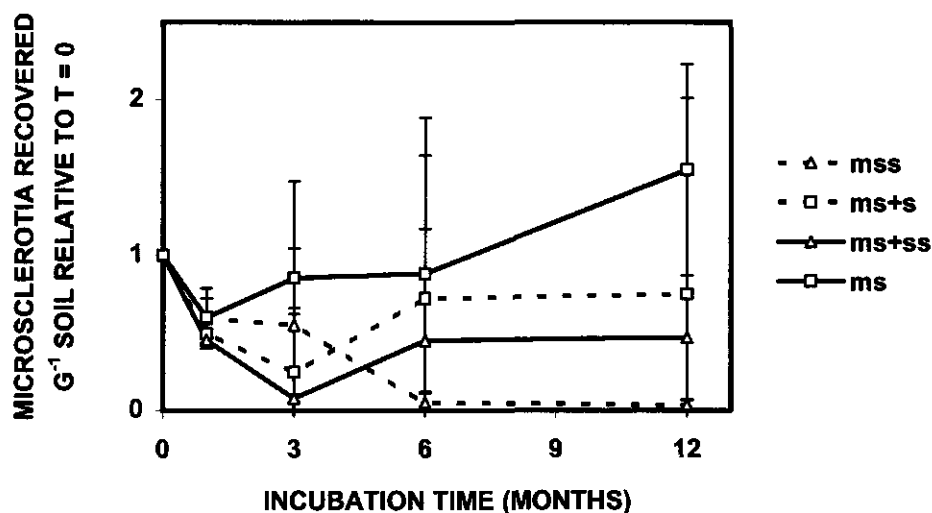


Figure 4.4. Effect of incorporation of plant material on the survival of microsclerotia of *Verticillium dahliae* in an unsterilised sandy field soil. The number of microsclerotia recovered g^{-1} air-dry soil relative to the number at $t = 0$ (= one day after start of the experiment) is shown. As inoculum, field-collected potato stems (FPS) containing microsclerotia was used. Treatments: mss = incorporation of 3 g of 1-cm-long FPS containing microsclerotia; ms+s = separation of microsclerotia from 3 g of FPS by grinding and sieving, followed by incorporation of both microsclerotia and FPS into the soil; ms+ss = separation of microsclerotia of 3 g of FPS by grinding and sieving, sterilisation of the ground FPS, followed by incorporation of both microsclerotia and the sterile FPS; ms = separation of microsclerotia of 3 g of FPS by grinding and sieving, followed by incorporation of the microsclerotia alone. Data for three different sources of inoculum were merged. Error bars indicate standard deviations of the means of the three inocula.

K1, K2, and K3 respectively. Inoculum sources K1-K3 also responded similarly to the different treatments and the data were combined. The treatment with intact pieces of potato stems incorporated into the soil resulted in a sharp decrease in recovery after 6 months; after 12 months the fungus could not be recovered at all (Figure 4.4). By contrast, density of microsclerotia recovered from soils where potato stem tissue was excluded remained high and increased to values higher than those found at the start of the experiment ($P = 0.10$).

Discussion

Very few microsclerotia were recovered one day after incorporation of the material into soil, varying between 5.5–31% for experiments 2 and 3. After 1–6 months, the recovery of microsclerotia decreased even further. Decreased recovery was also observed after 3 and 6 months for experiment 1 and after 1 and 3 months for experiment 4. This decrease was followed by an increase for most treatments to levels that were always lower than the number of microsclerotia incorporated into soil. This study was designed to evaluate the effect of temperature, moisture, and organic matter on the survival curve of microsclerotia of *V. dahliae*. Since formation of new microsclerotia in unsterile soil in the absence of plants is reportedly rare, we expected to observe only decreases in densities of microsclerotia in time. However, in all experiments, the numbers of microsclerotia recovered were in many cases equal to, or considerably higher than, previous harvests. The variation in recovery corresponds with results obtained by Wheeler and Rowe (1995), who reported strongly varying recoveries, mostly between 0 and 100%, depending on soil type and drying time of the soil samples, but the authors could not discover a certain pattern in the effect of drying on recovery. The variation in densities of microsclerotia found here may be due to real fluctuations in density by death or other events affecting the germination of microsclerotia on the agar medium. Germination and the subsequent formation of new microsclerotia are prerequisites for the recovery of a microsclerotium with the method used.

V. dahliae possesses no or only very limited competitive saprotrophic ability (Wilhelm, 1951), making the formation of new microsclerotia in unsterile soil in the absence of plant roots highly unlikely. Formation of new microsclerotia in soil in the absence of roots has been reported only after amendment of glucose to the soil (Menzies and Griebel, 1967; Emmatty and Green, 1969; Green and Papavizas, 1968). In our experiments no nutrients were added nor was the soil sterilized. In addition, formation of new microsclerotia in soils exposed continuously to -28°C (experiment 2) cannot have occurred and therefore cannot explain the apparent two-fold increase of microsclerotia after 3, 6, and 12 months. If the measurements of densities of microsclerotia in soil by Wheeler and Rowe (1995) reflected the amount of viable microsclerotia, it would have been impossible to measure 0% microsclerotia recovered at one sampling occasion and 100% at a subsequent date. Thus, we conclude it was unlikely that formation of new microsclerotia played a major role.

Evans *et al.* (1966) and Green (1980) observed an initial increase of inoculum density after incorporation of field-collected microsclerotia and attributed this to the dissociation of

microsclerotia that were initially clumped together in host tissue. Clumping of microsclerotia could not play a role in our experiments since in experiments 1 and 4 the material was ground before plating. In experiments 2 and 3 the inoculum consisted of material that had been ground and passed through a 106- μ m sieve. In addition, the inoculum was checked under the dissecting microscope prior to incorporation into soil for presence of clumps of microsclerotia.

The problems with quantifying *V. dahliae* population densities in soil are well-known (Wheeler and Rowe, 1995; Termorshuizen *et al.*, 1998). Recovery is generally 30–40% at best, depending on soil characteristics; it may vary considerably between soil sample replicates and complete failure of an assay may even occur (Termorshuizen *et al.*, 1998). Thus, low recovery rates could have been due to limitations in the detection procedure. However, sequential samples in the different experiments were taken independently using freshly prepared agar plates. We therefore conclude that the consistently observed low recoveries in the first two or three sampling occasions were not affected by random 'noise' of the detection procedure.

The observed variation in density of microsclerotia in time may also be caused by variation in germinability of viable microsclerotia. In other words, microsclerotia may acquire a reversible non-constitutive dormancy, which is released in time. This relates to the phenomenon of mycostasis, the inhibition of germination of fungal propagules in natural soils (Lockwood, 1977). Evidence exists that deficiency of nutrients needed for germination is a significant factor involved in this phenomenon (Lockwood, 1977). Thus, it may be postulated that micro-organisms resident on the surface of spores/microsclerotia consume exudates from the propagules of which a certain threshold concentration is necessary for their germination (Hsu and Lockwood, 1973; Bristow and Lockwood, 1975). In line with this hypothesis is the observation of Toyota and Kimura (1993), who isolated bacteria from the surface of chlamydospores of *Fusarium oxysporum* f.sp. *raphani* that inhibited germination but were not antagonistic to fungal growth *in vitro*. This phenomenon may occur on agar plates as well since the MSEA-medium used to plate soil samples is semi-selective only and antagonism may hinder the germination of microsclerotia. Bacteria resident on the surface of microsclerotia do not need to be affected by the antibiotic oxytetracyclin in the agar media, because the microsclerotia are spread on, and not into, the medium. Thus, as treatments and incubation time may affect the presence of the saprotrophic micro-organisms on the surface of microsclerotia, the ability of microsclerotia to germinate may vary accordingly. As such, variation in recovery of microsclerotia from soil may represent a technical problem related to the procedure used to quantify *V. dahliae* in soil. An indication that agar plating conditions are important is obtained from the observation that methods involving the plating of air-dry soil generally perform better than methods involving the plating of soil

suspensions (Termorshuizen *et al.*, 1998). Under dry plating conditions the activity of micro-organisms resident at the surface of microsclerotia is likely to be considerably lower than under wet conditions. The decrease in recovery of microsclerotia incubated in the absence of soil (experiment 1) may be related to the same phenomenon of mycostasis. The prevailing conditions of high humidity may have favoured the growth of micro-organisms resident in the potato stems. The difference in germination percentage of the starting material collected in a commercial potato field (varying between 67 and 89%), and that of the material incubated for 3 and 6 months may be caused by the fact that the starting material was air-dry when the microsclerotia were plated, whereas the microsclerotia that were plated later came directly from damp potato stems.

Low recovery rates were recorded consistently after 1 month of incubation in all experiments; and, except in experiment 2, for many treatments after 3 months of incubation. If the mycostasis hypothesis is correct, low recoveries would correspond to high numbers of micro-organisms on the surface of microsclerotia, caused by a relatively high leakage of nutrients by the microsclerotia. This would agree with the observation that exudates from sclerotia of *Sclerotium rolfsii* decrease exponentially during 26 days in the soil (Hyakumachi and Lockwood, 1989). At sampling occasions later than 3–6 months, leakage is likely declined to low levels, and populations of micro-organisms on the surface of microsclerotia may decrease. In natural soils, microsclerotia germinate only, or mainly, near roots (Ben-Yephet and Pinkas, 1977; Huisman and Gerik, 1989), where carbon supply is not limiting (Lynch and Whipps, 1990). The proportion of germinating microsclerotia in the rhizosphere may then depend on the amount of root exudation and competition with the rhizosphere microflora, including the mycostasis-inducing micro-organisms resident on the surface of microsclerotia.

Based on our results, little can be said about factors affecting the survival of microsclerotia of *V. dahliae*, since we were unable to distinguish between dead and non-germinable but viable microsclerotia. However, it is clear that nearly all treatments resulted in the survival for at least 12 or 24 (experiment 1) months of some fractions of the *V. dahliae* population. Thus we may conclude that a great portion of microsclerotia is not sensitive to temperatures between –28 and 25°C and pF 2.0–2.8, and fluctuations of these values in time. These observations are largely in line with those of Green (1980), who concluded that temperature (4 and 28°C) and soil moisture (pF 2.5 and 4.2) were not the main limiting factors in survival of *V. dahliae* over two years or more. In experiment 1, decrease of recovery continued throughout the experiment for the 25°C treatments, which may indicate a true decrease in survival. Incubation at 25°C and 75% relative humidity was the only treatment that resulted in no recovery at the final sampling occasion after 24 months. This may have been due to desiccation of

the microsclerotia. Pataky and Beute (1983) reported low survival rates of microsclerotia of *Cylindrocladium crotalariae*, the causal agent of black rot of peanut, when incubated on the soil surface compared to microsclerotia incorporated into the soil, and they attributed this to low soil matric potentials. More detailed studies on the recovery of microsclerotia of *V. dahliae* are necessary. Bioassays need to be performed with a short exposure time to determine whether the germinability of microsclerotia is affected in soil. For example, the infection density of *V. dahliae* on the roots of *Arabidopsis thaliana* could be determined (Chapter 2). Also, the study of the population of micro-organisms colonizing the surface of microsclerotia deserves more attention. Perhaps these organisms can be exploited to suppress the germination of microsclerotia in the rhizosphere of hosts.

CHAPTER 5

ABOVE-GROUND APPLICATION OF *TALAROMYCES FLAVUS* TO CONTROL *VERTICILLIUM DAHLIAE*

Summary

The antagonist *Talaromyces flavus* was applied onto potato stems infected with *Verticillium dahliae* and its effects on survival and inoculum potential of microsclerotia of the plant pathogen were evaluated. Three weeks after application of 3 g alginate prill formulation of *T. flavus* on 150 1-cm-long pieces of potato stems, the antagonist was growing prominently over the potato stems at 25°C but not at 15°C. Germinability of microsclerotia was reduced only in the three experiments carried out at 25°C and not in two experiments at 15°C. After 21 days of above-ground incubation, 15 potato stems were incorporated into a sand/potting soil mixture. Populations of *T. flavus* and *V. dahliae* were determined 4 and 10 months after incubation. The population level of *T. flavus* was significantly ($P < 0.05$) higher in the treatments where after its application the potato stems were left 21 days above-ground compared to direct incorporation of the stems + *T. flavus* into the soil. This was attributed to the relatively low levels of competition occurring above-ground. The population level of *T. flavus* persisted at at least 240 colony-forming units g⁻¹ soil, and showed a 10 to 12-times increase after 10 months compared to 4 months after incubation at 25°C. Population levels of *V. dahliae* were significantly decreased by *T. flavus* at both incubation temperatures. Leaving *T. flavus*-treated potato stems above-ground for 21 days did not affect the survival of microsclerotia compared to direct incorporation of the treated potato stems into the soil. A significant inverse relation between population size of *T. flavus* and that of *V. dahliae* was found only for incubation at 25°C. To determine the inoculum potential of *V. dahliae*, 4-week-old seedlings of *Arabidopsis thaliana* were planted either directly after incorporation of the potato stems in soil at 20°C; or soils were left another 21 days at 15 or 25°C before the bioassay with *A. thaliana* was carried out at 20°C. Temperature did not affect the development of senescence of *A. thaliana*, which was retarded significantly for all *T. flavus* treatments. This could be due to relatively high levels of *T. flavus* occurring in all experiments. It is concluded that application of *T. flavus* on fresh organic debris containing microsclerotia

reduces inoculum densities of *V. dahliae*. The effect of population density of *T. flavus* on the rate of development of wilt symptoms caused by *V. dahliae* warrants more research.

Introduction

Verticillium dahliae Kleb. is a soilborne pathogen causing wilting and early senescence in many crops (Pegg, 1974). The fungus can survive as microsclerotia in soil for periods up to 14 years (Wilhelm, 1955) in the absence of host plants. The disease cycle is monocyclic (Schnathorst, 1981): microsclerotia developing on senescent host tissue incite new infections only in a following crop. The number of microsclerotia produced on a certain host is therefore a reliable predictor for disease in a subsequent host crop (Mol, 1995d). Thus, any method that reduces formation of new microsclerotia will lessen future problems with verticillium wilt. Several methods to reduce soilborne inoculum of *V. dahliae* such as solarization (Katan *et al.*, 1976) have been advocated. Only few attempts were made to reduce the amount of microsclerotia on the standing crop, including removal or incorporation of infected crop debris (Mol *et al.*, 1995; Mol and Scholte, 1995b). Another approach would be to apply biocontrol agents above-ground to the newly formed microsclerotia.

Talaromyces flavus (Klöcker) Stolk & Samson is an antagonist of several sclerotium-forming pathogens including *V. dahliae*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *Sclerotium rolfsii* (Boosalis, 1956; Marois *et al.*, 1982; McLaren *et al.*, 1986; Powelson and Rowe, 1993; Madi *et al.*, 1997; Nagtzaam *et al.*, 1998). It has been shown to have potential in suppressing verticillium wilt in, *e.g.*, eggplant (Marois *et al.*, 1982) and potato (Fravel *et al.*, 1986; Nagtzaam, 1995). *T. flavus* is a common inhabitant of cultivated soils (Gams, 1992) and has been registered for control of verticillium wilt in horticultural crops, *e.g.*, strawberry and cucumber (Zeise, 1997; Koch, 1999).

The consistency of the biocontrol effect of *T. flavus* is reportedly low (Nagtzaam, 1998), which is often attributed to the low competitive ability of *T. flavus* in soil. Adams (1990) classified *T. flavus* as a passive mycoparasite that is generally inactive in natural soils. However, several research workers have reported active growth of *T. flavus* in the rhizosphere. Preference of *T. flavus* for the rhizosphere, notably the root tip, has been reported (Marois *et al.*, 1984), and can be regarded as a highly desirable trait, since the root tip is considered to be the main infection site of *V. dahliae* (Fitzell *et al.*, 1980; Gerik and Huisman, 1988). Fahima and Henis (1995) observed the spread of *T. flavus* to new roots and its presence on microsclerotia near the root surface. Populations of *T. flavus* were found to be larger in the rhizospheres of verticillium-

infected roots than in uninfected roots of eggplant (Fahima and Henis, 1995). On the other hand, Nagtzaam and Bollen (1997) observed a log-linear decrease of population size of *T. flavus* in the rhizosphere of eggplant and potato with increasing distance from *T. flavus*-coated seeds or tubers, suggesting a passive movement of the antagonist along the root. Densities of *T. flavus* were lower than 20 colony-forming units m⁻¹ root length at 9 cm or more distance from the stem base (Nagtzaam and Bollen, 1997).

The weak ability of *T. flavus* to colonize the soil and the inconsistent results concerning the ability of *T. flavus* to multiply in the rhizosphere raise the question whether current methods of application are efficient. When applied as seed coating, an antagonist needs to grow along the root to protect the plant against *V. dahliae* (Nagtzaam and Bollen, 1997). Root-dipping is advised frequently but is expensive and again the antagonist needs to accompany the growing root. An alternative approach could be to attack newly-formed microsclerotia. The majority of microsclerotia is formed above-ground in senescent shoot tissue (Mol and Scholte, 1995a). Thus if *T. flavus* can inactivate microsclerotia in such tissues before incorporation of the debris into the soil, competition with soil biota would be avoided. At the same time the antagonist would increase in density prior to incorporation into the soil. Spraying *T. flavus* directly onto microsclerotia can cause complete inactivation of *V. dahliae* (Fahima *et al.*, 1992).

The purpose of the present study was to test the effect of *T. flavus* by above-ground application to microsclerotia-containing potato stems followed by mixing the stems with the soil. The effects on population densities of the antagonist and the pathogen were recorded and the effect on the inoculum potential of the pathogen was studied in a bioassay.

Materials and methods

Dead potato stems containing microsclerotia of *V. dahliae* were collected from a potato field in the province of Drenthe (Experiments 1 and 3) or near Wageningen (Experiment 2) in October 1997 and November 1998 respectively. The potato stems were allowed to dry at room temperature, cut into 1-cm-long pieces, and stored dry at room temperature until further use.

T. flavus isolate Tf-1 was provided by Nagtzaam (Nagtzaam and Bollen, 1997) and originated from a sclerotium of *Sclerotinia minor* Jagger in the U.S.A. (Dunn and Lumsden, 1981). Inoculum of *T. flavus* alginate prill was produced as described by Fravel *et al.* (1985) and Lewis and Papavizas (1985). The fungus was grown in Petri dishes containing Potato-Dextrose Agar (PDA, Merck, Darmstadt, Germany) for 21 days at 30°C in the dark. Cleistothecia of *T.*

flavus were scraped gently from 3-week-old PDA plates, suspended in water, and crushed. The suspension was filtered through cheese cloth, the number of ascospores was determined with a haemocytometer and the suspension was diluted to reach 5.0×10^6 ascospores ml^{-1} . The viability of ascospores was determined by dilution plating onto PDA. Ten g of sodium alginate (Janssen Chimica, Belgium) and 110 g of powdered wheat bran were mixed with 500 ml of the suspension containing *T. flavus*. The mixture was dripped into a solution of 100 mM CaCl_2 where pellets of 2 mm formed by polymerization. The pellets were washed gently with tap water and allowed to dry on 2 layers of filter paper for 7 days at room temperature. After air-drying, the pellets were ground in a Retsch grinding mill (0.5 mm pore size) (Retsch, Haan, Germany) and stored at 4°C until further use. The air-dried formulation of *T. flavus* contained 10^5 ascospores g^{-1} .

One-cm-long pieces of air-dry potato stems (each about 0.05 g) containing microsclerotia were placed on a plastic screen table (height 6 cm) in a plastic box (l/w/h, 25/10/9 cm) containing a 3-cm layer of water to maintain a high relative humidity. Three grams of *T. flavus* alginate prill was then sprinkled dry by hand over 150 stems. A control treatment was sprayed with a twice autoclaved suspension of *T. flavus* alone (20 min 121°C; 24 h interval between the two sterilizations). The treatments were replicated five times. The stems were incubated in the dark at 15 or 25°C (experiments 1 and 2) or 25°C only for 21 days. After incubation, 25 microsclerotia collected from 5 randomly selected potato stem pieces were plated individually on Modified Soil Extract Agar (MSEA; Harris *et al.*, 1993) for every treatment. After 21 days of incubation at 23°C in the dark germination was assessed under the dissecting microscope.

Fifteen randomly selected stem pieces treated with *T. flavus* or with twice autoclaved inoculum of *T. flavus* that had been incubated at 25°C for 0 or 21 days were incorporated into a sand / potting soil mixture (2/1, v/v) at pH 2.0 in a 400 cm^3 pot (w/d/h, 7/7/8 cm). The survival of *V. dahliae* and *T. flavus* was assessed by incubating the pots at 15 or 25°C for 4 (experiment 1 and 2) or 10 (experiment 1) months and determining microsclerotium and ascospore densities respectively.

The density of microsclerotia of *V. dahliae* in soil was determined largely following the method described by Harris *et al.* (1993). In summary, 12.5 g of soil was air-dried for 14 days, and sieved over 106 and 20 μm screens. The material remaining on the 20 μm sieve was suspended in 50 ml 0.08% water agar and 0.8 ml was plated onto each of 10 Petri dishes containing MSEA (detection level 2 microsclerotia g^{-1} soil). Plates were incubated in the dark at 23°C for 21 days and subsequently the number of colonies of *V. dahliae* was counted. The density of ascospores of *T. flavus* in soil was determined according to Boosalis (1956). A 10-g soil sample was suspended in 50 ml 0.08% water agar, mixed well, and shaken for 30 min at 150 rpm

on a shaker (Gerhardt), followed by shaking for 30 min at 60°C at 150 rpm in a shaker bath (Grant Instruments Ltd., Barrington, Cambridge). One ml of the suspension was spread over a Petri dish containing PDA amended with 50 ppm oxytetracyclin using a glass rod. Petri dishes were dried in an airflow cabinet for 30 min before closure. The dishes were inverted and incubated at 30°C in the dark for 5 days and the number of colonies was determined.

The inoculum potential of *V. dahliae* was assessed in a bioassay with *Arabidopsis thaliana* ecotype Columbia (Chapter 2). This was done with soil samples immediately or 21 days after incorporation of the *T. flavus*-treated and untreated potato stems containing microsclerotia of *V. dahliae* in soil. Seeds were allowed to germinate by spreading them over humid potting soil at 23°C in a climate chamber and 21-day-old seedlings were transplanted to the test soils. One seedling was planted in every pot and 10 pots per treatment were prepared. The plants were grown in a climate chamber at 20°C with 16 h light (Philips TD 32W/84 HF); watering was done

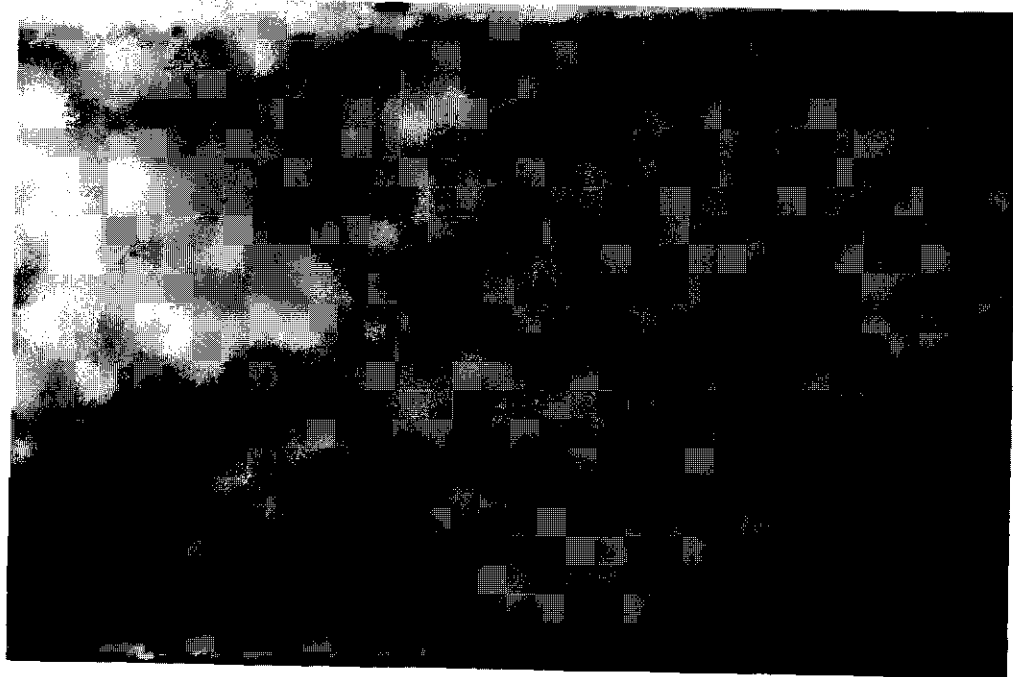


Figure 5.1. A dead potato stem containing microsclerotia of *Verticillium dahliae* 7 days after treatment with an alginate prill formulation of *Talaromyces flavus* and incubated at 100% relative humidity and 25°C.

by hand as necessary. Senescence of rosette leaves and stems was evaluated separately after 28 and 35 days, respectively, by using senescence indices (SI) ranging from 0 to 5. For the rosette leaves: 0 = 0-5% of the rosette leaves showing some necrosis; 1 = 6-15%; 2 = 16-25%; 3 = 26-50%; 4 = 51-75%; and 5 = 76-100%. For the stems: 0 = no necrosis; 1 = stems still green but 1-5% of leaves showing necrosis; 2 = 1-5% of the stems brownish or yellowish, but 6-25% of the leaves showing necrosis, and 1-5% of siliquae and/or flowers yellowish or brownish; 3 = 6-25% of the stems brownish or yellowish, 26-75% of the leaves showing necrosis, and 6-25% of the siliquae brownish; 4 = 26-50% of the stems brown, 26-100% of the leaves showing necrosis, 26-50% of the siliquae brown and 1-75% of the flowers yellow or brown; and 5 = most of all stems, leaves, flowers, and siliquae showing necrosis.

Analysis of variance was applied (SPSS for MS Windows Release 6.1) after log-transforming the data ($\log_{10}(x+1)$), because of homoscedasticity. Treatment means were separated by the Tukey-HSD at $P < 0.05$. Differences in survival of *T. flavus* and *V. dahliae* between two sampling occasions (Experiment 1) were compared by analysis of variance of the two temperature treatments separately.

Table 5.1. Germination of microsclerotia of *Verticillium dahliae* 21 days after spraying an ascospore suspension of *Talaromyces flavus* onto dead potato stems containing microsclerotia of *Verticillium dahliae*.

<i>T. flavus</i>	Experiment 1		Experiment 2		Experiment 3
	15°C	25°C	15°C	25°C	25°C
No	40 a ^{1,2}	56 a	47 a	64 a	74 a
Yes	23 a	36 b	42 a	36 b	14 b

¹ Data shown are back-transformed values after arcsine-root transformation.

² Within each column, values followed by the same letter in a column do not differ significantly, according to Tukey's HSD test ($P < 0.05$).

Results

Effect of applying T. flavus over potato stems containing microsclerotia of V. dahliae

At 25°C, ascospores of *T. flavus* in alginate prill germinated 4-7 days after sprinkling over potato stems containing microsclerotia. The fungus formed initially yellowish white, later yellow, mycelium over the stems after incubation (Figure 5.1). At an incubation at 15°C, activity of *T. flavus* could not be observed at 25 × magnification. Germination decreased significantly ($P < 0.05$) for microsclerotia incubated at 25°C, but not significantly at 15°C (Table 5.1).

Survival of T. flavus and V. dahliae in soil

T. flavus survived a period of 10 months in soil at levels varying between 2.4×10^2 and 6.6×10^3 ascospores g⁻¹ dry soil (Table 5.2). The density of ascospores was significantly ($P < 0.05$) increased in soil 1.5 to 2.4 times when the potato stems after treating with *T. flavus* were left above-ground for 21 days. Survival was significantly ($P < 0.05$) higher at 25°C than at 15°C. After 4 months of incubation, the population of *T. flavus* was 1.7 to 2.7 times higher at 25°C than

Table 5.2. Survival of ascospores of *Talaromyces flavus* in soil after an alginate prill formulation onto potato stems containing microsclerotia of *Verticillium dahliae* and incorporation into soil for 4 or 10 months (experiment 1).

Temperature (°C)	Incubation above-ground ¹	<i>T. flavus</i>	Numbers of ascospores g ⁻¹ dry soil	
			4 months	10 months
15	No	No	0 ²	0
15	No	Yes	240a ³	265a
15	Yes	No	0	0
15	Yes	Yes	350ab	640b
25	No	No	0	0
25	No	Yes	399ab	3068c
25	Yes	No	0	0
25	Yes	Yes	961b	6628c

¹ Potato stems treated with *T. flavus* were immediately mixed with soil ('No') or left 21 days above-ground at 25°C and 100% relative humidity before incorporation into the soil and placement at 15 or 25°C ('Yes').

² Data shown are back-transformed averages from log₁₀(x+1) values.

³ Within each column, values followed by the same letter in a column do not differ significantly, according to Tukey's HSD test ($P < 0.05$).

at 15°C, and this difference increased 10 to 12 times after 10 months of incubation ($P < 0.01$).

The density of microsclerotia in soil was subject to a large variation, probably due to the variation in the numbers of microsclerotia in the starting material. There was a main effect of *T. flavus* leading to a significant ($P < 0.01$) reduction of *V. dahliae* at all sampling occasions, especially at 25°C (Table 5.3). The data suggest that marked reduction of *V. dahliae* occurs especially at population densities of *T. flavus* < 1000 ascospores g^{-1} soil. No significant correlation was found between population densities of *T. flavus* and *V. dahliae* across all individual pots. However, when considering only the 25°C pots, a significant negative correlation ($r = -0.64$; $P < 0.001$) was obtained.

Bioassay

Disease indices increased steadily as the plants grew older. Test plants developed slower senescence in the *T. flavus*-treatments compared to the controls (Figure 5.2). Effects of

Table 5.3. Effect of applying *Talaromyces flavus* formulated into alginate prill onto potato stems containing microsclerotia of *Verticillium dahliae* followed by incorporation of the potato stems into soil at either 15 or 25°C on the density of *Verticillium dahliae* g^{-1} dry soil after 4 or 10 months of incubation.

Temperature (°C)	Incubation above-ground ¹	<i>T. flavus</i>	Number of microsclerotia g^{-1} dry soil		
			Experiment 1		Experiment 2
			4 months	10 months	4 months
15	No	No	1049 b ^{2,3}	761 c	962 bc
15	No	Yes	415 ab	962 c	457 ab
15	Yes	No	831 ab	1479 c	791 abc
15	Yes	Yes	340 ab	399 bc	451 ab
25	No	No	1347 b	559 bc	989 c
25	No	Yes	103 a	25 a	434 a
25	Yes	No	667 ab	341 bc	1368 c
25	Yes	Yes	245 ab	76 ab	948 bc

¹Potato stems treated with *T. flavus* were either immediately mixed with the soil ('No') or left 21 days above-ground at 25°C and 100% relative humidity before incorporation into the soil and placement at 15 or 25°C ('Yes').

²Data shown are back-transformed averages from $\log_{10}(x+1)$ values.

³ Within each column, values followed by the same letter in a column do not differ significantly, according to Tukey's HSD test ($P < 0.05$).

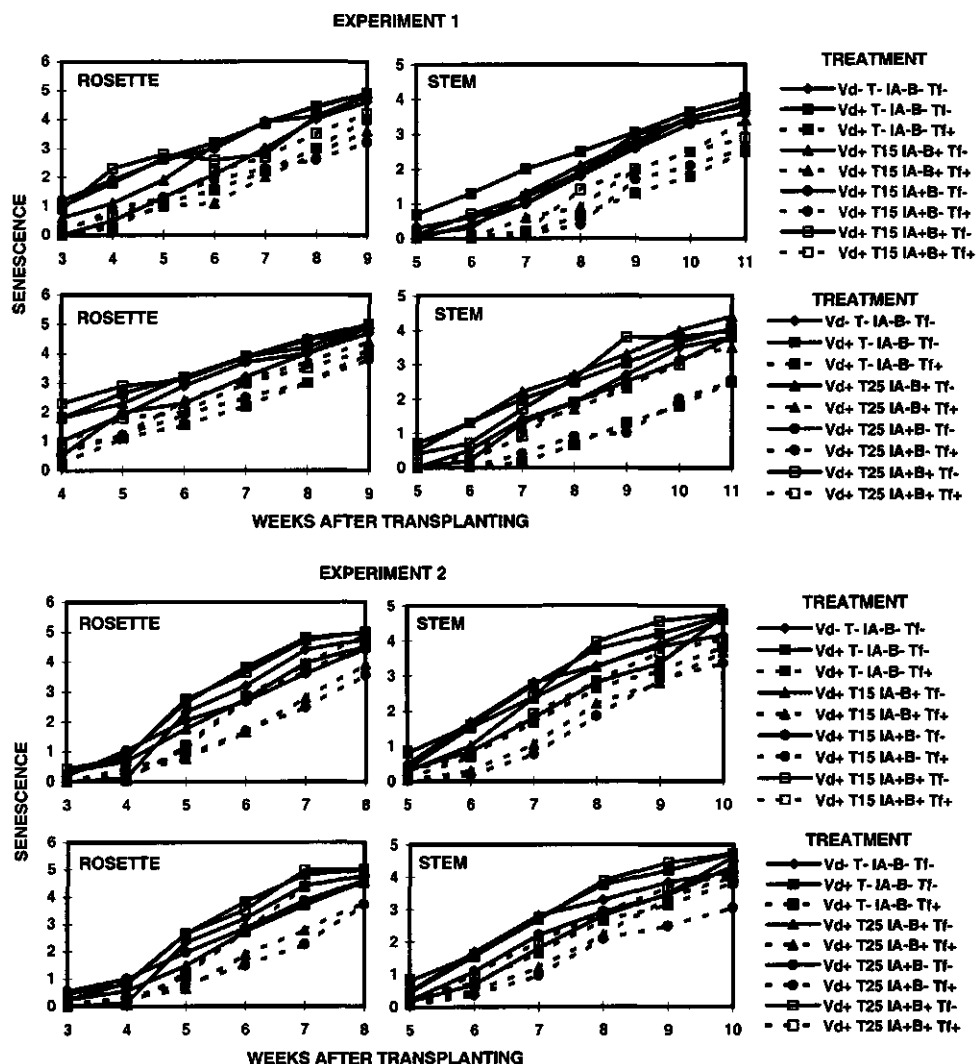


Figure 5.2. Development of senescence of *Arabidopsis thaliana* growing on differently treated soils. Vd- = soil not infested with *Verticillium dahliae*; Vd+ = soil infested with *V. dahliae*. Tf-/Tf+ = potato stems containing microsclerotia of *V. dahliae* treated with a twice sterilized or viable alginate prill formulation of *Talaromyces flavus*; IA-/IA+ = potato stems containing microsclerotia after treatment with *T. flavus* immediately incorporated in soil (IA-) or incubated above-ground at 100% relative humidity for 21 days; B-/B+ = after incorporation of the potato stems into soil, seedlings of *A. thaliana* were either planted immediately (B-) or 21 days later (B+); T-/T15/T25 = treated or untreated seedlings of *A. thaliana* were either grown immediately at 20°C (T-), or in the soil incubated first at 15°C (T15) or 25°C (T25) before carrying out the bioassay at 20°C. For an explanation of the senescence index, see text.

temperature, additional aboveground and/or belowground incubation prior to performing the bioassay gave no consistent effects on the area-under-the-disease-progress curve (Figure 5.2, Table 5.4). Dry weight values of the test plants were highly variable and not significantly different between treatments (results not shown).

Discussion

Talaromyces flavus consistently reduced microsclerotial density of *V. dahliae* in soil resulting in a slower rate of senescence in *A. thaliana*. At 25°C the ascospore population of *T. flavus* increased considerably with time, on average from 6.8×10^2 to 4.8×10^3 colony-forming units g⁻¹ soil, indicating active growth in soil. Thus, although active growth of *T. flavus* in soil may not be common (Adams, 1990), it may occur if large amounts of host material are together, as was the case in our experiments, where 1-cm-long potato stem pieces treated with *T. flavus* were left intact following incorporation into soil. When potato stems treated with *T. flavus* were left above-ground for 21 days, populations of *T. flavus* were higher than when the potato stems were incorporated into the soil directly after treatment. A much lower level of competition on the potato stems can be assumed in the absence of soil.

The density of microsclerotia in soil was significantly reduced by *T. flavus* (Table 5.3). For nearly all treatments, there was no effect of leaving *T. flavus*-treated stems 21 days above-ground (Table 5.1), indicating that inactivation of microsclerotia by *T. flavus* occurred primarily below-ground. An alternative explanation is that, above-ground, some microsclerotia were weakened by *T. flavus*, and subsequently killed in soil by the continued action of *T. flavus*. At 25°C, a high population density of *T. flavus* occurred together with a decline of *V. dahliae*, supporting an active below-ground participation of *T. flavus* in the inactivation of microsclerotia. At densities of *T. flavus* higher than 1000 ascospores g⁻¹ soil, a consistent low density of microsclerotia was found.

T. flavus is known to have a high optimum temperature for growth (Nagtzaam, 1998) and our experiments confirm this; active growth occurred at 25°C but not or only sparsely at 15°C. It is therefore surprising that *T. flavus* incubated at 15°C gave a reduction in the rate of senescence of *A. thaliana* that was not different from that observed at 25°C. A similar effect was observed by Nagtzaam and Bollen (1997) who found no significant difference in the colonization of eggplant and potato roots incubated at 15 or 20°C for an isolate of *T. flavus* that *in vitro* grew 30% faster at 20°C than at 15°C. In addition, they even were able to recover *T. flavus* from young roots of

Table 5.4. Effect of applying a *Talaromyces flavus* - alginate prill formulation onto potato stems containing microsclerotia of *Verticillium dahliae* followed by incorporation into soil at 15 or 25°C and planting seedlings of *Arabidopsis thaliana* on area-under-the-senesence-progress curve assessed for rosettes and stems separately.

<i>V. dahliae</i>	Temperature (°C)	Incubation		<i>T. flavus</i>	Area-under-the-disease-progress curve			
		Above-ground ¹	Below-ground ²		Experiment 1		Experiment 2	
					Rosette	Stem	Rosette	Stem
No	20	No	No	No	13.2bcde ^{3,4}	11.4def	11.0cd	8.9defg
Yes	20	No	No	No	18.9f	14.9ef	14.7e	10.6g
Yes	20	No	No	Yes	10.0ab	5.1a	9.9bc	6.7bcd
Yes	15	No	Yes	No	17.1def	11.8def	11.5cde	8.6defg
Yes	15	No	Yes	Yes	8.9def	7.6c	7.4def	4.9ab
Yes	15	Yes	No	No	18.4f	11.2de	11.2cde	7.1bcde
Yes	15	Yes	No	Yes	10.6ab	5.5abc	6.9a	4.2a
Yes	15	Yes	Yes	No	17.4ef	12.2def	14.6de	10.3efg
Yes	15	Yes	Yes	Yes	12.5bcd	7.5bc	10.4c	7.3cdef
Yes	25	No	Yes	No	18.6f	15.9f	10.8cd	7.0bcd
Yes	25	No	Yes	Yes	12.7bcde	10.0cd	7.4ab	5.6abc
Yes	25	Yes	No	No	16.2cdef	10.9de	12.1cde	7.9cdefg
Yes	25	Yes	No	Yes	11.0bcde	5.5ab	6.6a	4.5ab
Yes	25	Yes	Yes	No	19.7f	14.8ef	14.7de	10.5fg
Yes	25	Yes	Yes	Yes	13.4bcdef	10.3cd	10.7cd	7.6cdefg

¹Potato stems treated with *T. flavus* were either immediately mixed with the soil ('No') or left 21 days above-ground at 25°C and 100% relative humidity before incorporation into soil and placement at 15 or 25°C ('Yes').

²The bioassay with *A. thaliana*, carried out at 20°C, was started directly after incorporation of the treated potato stems into the soil ('No') or 21 days later ('Yes'). Soil samples were incubated before start of the bioassay at 15 or 25°C.

³Data shown are back-transformed averages from $\log_{10}(x+1)$ values.

⁴Within each column, values followed by the same letter in a column do not differ significantly, according to Tukey's HSD test ($P < 0.05$).

eggplants grown at 12.5°C. Nagtzaam *et al.* (1998) obtained significant suppression of *V. dahliae* at 15°C at a population level of *T. flavus* of 0.1% (w/w) or higher. We applied a slightly lower amount of *T. flavus* inoculum of 0.063% (w/w). Nagtzaam and Bollen (1997) concluded that temperature may have a complex effect on root colonization influencing not only *T. flavus* directly but also other soil biota. In our experiments, all bioassays were performed at 20°C. Therefore, instead of exposing bioassay plants to different temperatures as carried out by Nagtzaam and Bollen (1997), any temperature effect measured in our bioassay relates to a difference in development of *T. flavus* at the time the soils were incubated, without test plant, at 15 or 25°C. So, the 'complex effects' exerted by temperature on root colonization as mentioned by Nagtzaam and Bollen (1997) may also be present in bulk soil. Alternatively, *T. flavus* may have had such high population densities that the maximum possible effects on disease development were obtained in all treatments. In addition, *T. flavus* may induce resistance at relatively low population levels, as suggested by Nagtzaam and Bollen (1997).

The considerable effect of *T. flavus* on the area-under-the-disease-progress curve was quite remarkable, given the high numbers of microsclerotia (c. 1400 g⁻¹ soil) incorporated into the soil. Although this number may seem high, the amount of potato stems incorporated into soil in our experiments (0.18% w/w) was based on realistic calculations. The high densities of microsclerotia measured may reflect the effect of grinding of soil samples before sieving and plating. Thus, clumping of microsclerotia, which in the field causes low initial estimates for inoculum density (Evans *et al.*, 1966; Mol *et al.*, 1996b), could not occur in our experiment.

There are several options for developing an integrated management system against verticillium wilt. They include tolerant cultivars (Paplomatas *et al.*, 1992) or cultivars accumulating low numbers of microsclerotia (Davis *et al.*, 1983), and rotating with non-hosts (Bollen *et al.*, 1989; Xiao *et al.*, 1998); soil infestations can also be managed through soil solarization in the Mediterranean climate zone (Katan *et al.*, 1976; Pullman *et al.*, 1981) or by biological soil disinfestation (Blok *et al.*, 2000) in areas not allowing the control of pathogens in soil by solar heat. Both control methods involve the tarping of soil with plastic. Tjamos and Fravel (1995) showed that the incorporation of *T. flavus* into soil can lead to increased inactivation of *V. dahliae*. Our results indicate that there is also scope for the application of *T. flavus* onto *Verticillium*-infected organic debris followed by soil solarization or biological soil disinfestation.

CHAPTER 6

CONTROL OF *VERTICILLIUM DAHLIAE* IN *ARABIDOPSIS THALIANA* AND EGGPLANT (*SOLANUM MELONGENA*) BY COMBINATION OF *PSEUDOMONAS* *FLUORESCENS* AND *TALAROMYCES FLAVUS*

Summary

The effects of *Pseudomonas fluorescens* and *Talaromyces flavus* on *Verticillium dahliae* and verticillium wilt of *Arabidopsis thaliana* and eggplant were studied. *P. fluorescens* strain P60 inhibited mycelial growth of 20 isolates of *V. dahliae* *in vitro* by at least 50% and also the formation of microsclerotia was significantly reduced by P60 for the majority of the isolates tested. In bioassays, *V. dahliae* significantly accelerated senescence of *A. thaliana* and eggplant. However, when plants were treated with P60, senescence progressed at the same rate as in the treatment without *V. dahliae*. Although treatment of plants with P60 did not lead to major reductions in the percentage of infected shoots of *A. thaliana*, the amount of microsclerotia produced by *V. dahliae* on senesced shoots was significantly reduced (up to almost 8-fold) in all four experiments. *P. fluorescens* could not be isolated from the interior of the host tissue, but it was present in the rhizosphere of both *A. thaliana* and eggplant up to 12 weeks after inoculation at densities ranging from 1.4×10^3 - 8.0×10^5 cfu g⁻¹ root tissue. Combination of P60 with *T. flavus* further improved control of *V. dahliae*. Compared to the control treatment, the amount of microsclerotia formed was reduced up to almost 40-fold by combined application of P60 and *T. flavus*, and control obtained by the combination was significantly better than the reductions obtained by applications of each agent separately.

Introduction

Verticillium dahliae Kleb. causes wilt in a wide variety of dicotyledonous crops such as cotton, potato, rose, several nursery trees (maple, ash), olive, and eggplant. *V. dahliae* forms microsclerotia that enable the pathogen to survive for many years and to withstand adverse environmental conditions. Microsclerotia play a key role in the life and disease cycle of this vascular pathogen as they serve as survival structures and sources of inoculum (Schnathorst,

1981). Currently, no environmentally benign methods are available to manage verticillium wilt in intensive agriculture in temperate climates. In warmer climates, control by solarization is advocated (Katan, 1981; Melero-Vara *et al.*, 1995). Tolerant crops are only available for tomato and cotton. Tolerance to *V. dahliae* is also present in other crops such as potato, but presently these potato cultivars are not widely used due to the requirements of the processing industry and consumers.

Several attempts have been undertaken to apply antagonistic micro-organisms to control verticillium wilt. Biocontrol agents may parasitize or inactivate the resident microsclerotia and/or protect the host by limiting root infection. Parasitism or inactivation of resident microsclerotia may be successful only for polyphagous antagonists, given the generally low densities of *V. dahliae* in soil. Application of mycoparasites has been attempted with *Trichoderma* spp. (Ordentlich *et al.*, 1990; Georgieva, 1992) and *Gliocladium* spp. (Keinath *et al.*, 1991), but the results obtained so far were not satisfactory enough. Rhizosphere-colonizing antagonists seem to show more promising results. Among them, *Talaromyces flavus* has been registered for control of verticillium wilt in oil-seed rape and other horticultural crops (Zeise, 1997; Koch, 1999). In addition, several promising bacterial species, such as *Stenotrophomonas maltophilia* (Berg *et al.*, 1994), *Bacillus subtilis* (Podile *et al.*, 1985; Safiyazov *et al.*, 1995), and *Pseudomonas fluorescens* (Leben *et al.*, 1987; Safiyazov *et al.*, 1995) have been reported as potential antagonists of *V. dahliae*.

Combined applications of different biocontrol agents are expected to give more consistent positive results than single agents. Several studies reported an improvement of disease suppression of soilborne pathogens when different antagonists were combined (Park *et al.*, 1988; Bin *et al.*, 1991; Fuchs and Défago, 1991; Lemanceau and Alabouvette, 1991). For example, Bin *et al.* (1991) showed that application of *P. fluorescens* strain 2-79RN₁₀ led to an increased percentage of sclerotia of *Sclerotinia sclerotiorum* colonized by *Trichoderma* spp. compared to applying *Trichoderma* spp. alone. Combination of *Bacillus pumilus*, *B. subtilis*, and *Curtobacterium flaccumfaciens* provided better and more consistent control of three cucumber pathogens (Raupach and Kloepper, 1998) than single agents.

In this study, we investigated the effect of *P. fluorescens* strain P60 on verticillium wilt in *Arabidopsis thaliana* and eggplant. *A. thaliana* has been shown to be a sensitive and valuable bioassay plant for *V. dahliae* (Chapter 2). Control of *V. dahliae* by *P. fluorescens* P60 was studied in two types of soil, a potting soil and a field soil. The potential to increase the level of control of *V. dahliae* by combination of different antagonistic microorganisms was studied by combining P60 with *Talaromyces flavus* isolate R1.

Materials and methods

Isolates and production of inoculum

Twenty monoconidial isolates of *V. dahliae* collected from different host plants and different locations in the Netherlands (Table 6.1) were used in sensitivity tests with three isolates of *P. fluorescens*.

Microsclerotial inoculum was prepared from field-collected potato stems that contained microsclerotia. The potato stems were ground and successively sieved over screens with meshes of 106 and 20 μm respectively. The residue remaining on the 20 μm sieve was suspended in water containing 0.08% agar. The agar was added to obtain a suspension in which the microsclerotia were distributed homogeneously. The number of microsclerotia in the suspension was determined

Table 6.1. Origin of isolates of *Verticillium dahliae* used in this study.

No.	Host plant	Organ/Part	Location	Year
K3	<i>Solanum tuberosum</i> L.	Stem	Veenhuizen	1997
G3	<i>S. tuberosum</i>	Potato soil	Wageningen	1994
717.96	<i>S. tuberosum</i>	stem	Rolde	1991
C2	<i>S. tuberosum</i>	stem	Wageningen	1996
C3	<i>S. tuberosum</i>	stem	Wageningen	1996
V1	<i>S. tuberosum</i>	stem	Nieuweroord	1996
V3	<i>S. tuberosum</i>	stem	Nieuw-Balinge	1996
V4	<i>S. tuberosum</i>	stem	Bruntinge	1996
ES120	<i>Fraxinus excelsior</i> L.	wood	Lelystad	1988
A59	<i>Rubus fruticosus</i> L.	stem	Geldermalsen	1996
S12.2	<i>Syringa vulgaris</i> L.	wood branch	Aalsmeer	1996
AplatI	<i>Acer platanoides</i> L.	wood branch	Grubbenvorst	1993
A56	<i>Rosa</i> sp.	stem	Elst	1996
A45	<i>Fragaria</i> sp.	stem	Elst	1996
A60	<i>Acer</i> sp.	stem	Swolgen	1993
A63	<i>Forsythia</i> sp.	stem	Aalsmeer	1997
A17.1	<i>Phlox</i> sp.	stem	Gendt	1991
A61	-	soil	Lelystad	1997
A40	<i>Ribes rubrum</i> L.	stem	Geldermalsen	1996
C1	<i>Linum usitatissimum</i> L.	stem	Netherlands	1995

in small subsamples using a dissecting microscope at $25\times$ magnification. To determine germinability, 25 microsclerotia were plated individually on Petri dishes containing Modified Soil Extract Agar (MSEA) media (Harris *et al.*, 1993) by using an insect pin 000 (Emil Arlt, Australia). The Petri dishes were incubated up-side down in the dark at 23°C for 14 days, before the number of germinated microsclerotia was counted.

Pseudomonas fluorescens strains JMP22, P32, and P60, provided by Dr J.M. Raaijmakers (Laboratory of Phytopathology, WAU), were isolated from rhizosphere roots of wheat grown in a soilcropped continuously to wheat for 14 (JMP22) and 27 (P32 and P60) years, respectively, in 1998. The strains were cultured on King's medium B (KMB) agar (Merck, 64271 Darmstadt, Germany) at 25°C for 48 h. Spontaneous rifampicin-resistant (rif) mutants of JMP22, P32, and P60 were prepared as follows: *P. fluorescens* was suspended in 5 ml of sterile water to obtain 10^7 cells ml^{-1} . One hundred μl of the suspension was plated onto KMB agar supplemented with cycloheximide (100 mg l^{-1}) and rifampicin (100 mg l^{-1}). After incubation at 25°C for 48 h, single colonies of the strains appearing on the agar were transferred successively for three times to the same antibiotic-containing medium. Single colonies of the strains from the last plating were stored in a mixture of 1 ml of 80% glycerol and 1 ml of Luria-Bertani's solution (LB) containing 10 g l^{-1} NaCl, 10 g l^{-1} bacteriological peptone (Oxoid), and 5 g l^{-1} yeast extract (Oxoid) (Sambrook *et al.*, 1989). The stability of rif-resistance was tested by subculturing on KMB three times at 25°C for 48 h, followed by plating on KMB with and without cycloheximide and rifampicin. Rif-resistance was considered stable if the numbers of colony-forming units were not different on these two media.

Isolates R1 (Prophyta Biologischer Pflanzenschutz GMBH, Rostock, Germany) and Tf1 of *T. flavus* were used in this experiment. Isolate R1 originated from a sclerotium of *Sclerotinia minor* Jagger in the U.S.A. (Dunn and Lumsden, 1981). Isolate R1 was formulated onto an organic carrier as a water-dispersible granule containing 5×10^9 ascospores g^{-1} granule (Kersten, 1997). Granules of this isolate with the recommended density of 1.0×10^4 ascospores g^{-1} dry soil were prepared by thoroughly mixing an ascospore suspension with the soil used. Isolate Tf1, provided by Nagtzaam (Nagtzaam and Bollen, 1997), was cultured on Potato-Dextrose Agar (PDA, Merck, Darmstadt, Germany). It was formulated in alginate prill as described by Fravel *et al.* (1985) and Lewis and Papavizas (1985). Cleistothecia of *T. flavus* were scraped gently from 3-week-old PDA cultures, suspended in water, and crushed. The suspension was filtered through cheese cloth, the number of ascospores was determined with a haemocytometer, and the suspension was diluted to reach 10^7 ascospores ml^{-1} suspension. The viability of spores was determined by dilution plating onto PDA. Ten g of sodium alginate (Janssen Chimica, Belgium)

and 110 g of powdered wheat bran were mixed with a 500 ml aqueous suspension containing 10^9 ascospores in total. The mixture was dripped into a solution of 100 mM CaCl_2 where pellets formed by polymerisation. The pellets were then washed gently with tap water and allowed to dry on two layers of filter paper for 7 days at room temperature. After air-drying, the pellets were ground in a Retsch grinding mill (0.5 mm pore size) (Retsch, Haan, Germany) and stored at 4°C until further use. The number of viable propagules was assessed by plating 30-40 particles onto plates with Marois medium, incubated for 5 days at 30°C in the dark, and counted under a binocular microscope. The inoculum contained 4.5×10^7 ascospores g^{-1} pellets and 2.6×10^6 particles g^{-1} dry pellet.

In vitro assays

Strains P60, P32 and JMP22 of *P. fluorescens* were spot-inoculated on PDA or 1/5 strength of Potato-Dextrose Broth (PDB; Difco, Detroit, USA) amended with 15 g Technical Agar No. 3 (Oxoid, Basingstoke, England) at a distance of 1 cm from the edge of a Petri dish (diam. 9 cm). After incubation at 25°C for 24 h, a 5-mm plug of *V. dahliae* was placed in the middle of the dish. The Petri dishes were incubated upside down in the dark at 20°C for 25 days, and all treatments were replicated five times. Radial growth of *V. dahliae* was measured when the control dishes were almost covered. The amount of microsclerotia formed by *V. dahliae* was scored semi-quantitatively (0 = no microsclerotia, 1 = microsclerotia covering < 25% of the colony, 2 = 25-75%, and 3 = > 75%). The experiment was carried out three times.

Inhibition of *T. flavus* Tf1 and R1 by P60 was tested as described above for *V. dahliae*. The plates were incubated at 25 or 30°C, for 14 days. The experiment was carried out twice.

In planta assays

Colonization of the rhizosphere by Pseudomonas fluorescens. Seedlings of *Arabidopsis thaliana* (L.) Heyhn. ecotype Columbia and eggplant (*Solanum melongena* L.) cv. Black Beauty were grown in sieved potting soil. Soil collected from a field on the experimental farm 'Meterikse Veld' at Horst (Limburg, the Netherlands) which was a loamy sand with a pH-KCl of 6.5 and an organic matter content of 2.3% was also used. Four or three-week-old seedlings of *A. thaliana* or eggplant, respectively, were root-dipped in either a bacterial suspension of 10^7 cells ml^{-1} for 3 min or sterile water, blotted on sterile filter paper to remove excess water and planted in 10 replications in small plastic pots (w/w/h, 7/7/8). Incubation took place in a climate chamber at 20°C with 16 h light (Philips TD 32W/84 HF) and 8 h dark, and watering was done by hand as necessary. At 1, 4, 8, and 12 weeks after inoculation roots plus rhizosphere soil were collected by gently shaking the roots to remove loosely adhering soil. One gramme of root material including

rhizosphere soil was suspended in 5 ml sterile water and shaken vigorously for 1 min on a Vortex mixer, sonicated for 1 min. in an ultrasonic cleaner (Bransonic 12), shaken again on a Vortex mixer for 1 min and serially diluted. Fifty μL of every dilution were spread onto KB amended with cycloheximide (100 mg l^{-1}) and rifampicin (100 mg l^{-1}). Presence of *P. fluorescens* P60 in the interior of the plant was checked by plating 1-cm-long stem pieces either after surface sterilization in 1% NaClO for 30 sec, washing twice in sterile water, blotting dry on sterile filter paper, and plating on KB containing rifampicin and cycloheximide or by first grinding in a sterile mortar, adding 5 ml sterile water, and plating 1 ml of this suspension on the same medium. All plates were incubated at 25°C and colonies were enumerated after 72 h.

Effect of P. fluorescens on verticillium wilt. Seedlings were grown as described above. The following treatments were included: (1) Half of the pots with either loamy sand or potting mixture were infested with 50 germinable microsclerotia of *V. dahliae* isolate A17.1 g^{-1} dry soil; (2) root-dipping for 3 min of 4 or 3-week-old seedlings of *A. thaliana* or eggplant, respectively, in a bacterial suspension of *P. fluorescens* P60 of 10^7 cells ml^{-1} , or water; (3) planting the seedlings in loamy sand, described above, or potting soil mixed with sand (2:1, v/v). The treatments were replicated 5 times. Seedlings were grown under controlled conditions as described above. Senescence was evaluated for rosette leaves and stems (including stems, stem leaves, flowers, and siliquae) separately by using a senescence index (SI) ranging from 0 to 5. For the rosette leaves: 0 = 0-5% of the rosette leaves showing some necrosis; 1 = 6-15%; 2 = 16-25%; 3 = 26-50%; 4 = 51-75%; and 5 = 76-100%. For the stems: 0 = no necrosis; 1 = stems still green but 1-5% of leaves showing necrosis; 2 = 1-5% of the stems brownish or yellowish, 1-25% of the leaves showing necrosis, and 1-5% of siliquae and/or flowers yellowish or brownish; 3 = 6-25% of the stems brownish or yellowish, 26-75% of the leaves showing necrosis, and 6-25% of the siliquae brownish; 4 = 26-50% of the stems brown, 26-100% of the leaves showing necrosis, 26-50% of the siliquae brown, and 1-75% of the flowers yellow or brown; and 5 = most of all stems, leaves, flowers, and siliquae showing necrosis. The *A. thaliana* test plants were harvested after total senescence, and the amount of newly formed microsclerotia in the shoot was determined as follows. Microsclerotia were separated from shoot tissue by grinding and sieved over screens with meshes of 106 and $20 \mu\text{m}$ respectively. The residue remaining on the $20 \mu\text{m}$ sieve was suspended in water containing 0.08% agar. The agar was added to obtain a suspension in which the microsclerotia were distributed homogeneously. The number of microsclerotia in the suspension was determined in small subsamples using a dissecting microscope at $25\times$ magnification. The infection percentage of *V. dahliae* in eggplant was determined by plating 1-cm-long pieces of plant stems. The stem pieces were placed on Ethanol Agar after surface

sterilization in 1% NaClO for 30 sec, washing twice in sterile water, and blotting dry on sterile filter paper. The whole experiment was carried out in duplicate.

Combination of P. fluorescens and T. flavus. The following treatments were included: (1) Infestation of the soil with 50 germinable microsclerotia g⁻¹ dry soil of isolates AplatI or ES120, or not; (2) root-dipping for 3 min of 4-week-old seedlings of *A. thaliana* in a bacterial suspension of *P. fluorescens* P60 of 10⁷ cells ml⁻¹ suspension, or water; (3) planting the seedlings in loamy sand mixed with *T. flavus* isolate R1 at a density of 1.0 × 10⁴ ascospores g⁻¹ of soil, or without *T. flavus*; (4) exposing the seedlings to *P. fluorescens* P60 and *T. flavus* R1 jointly at full doses as described above or at halved doses (*i.e.* 5 × 10⁶ cells ml⁻¹ suspension and 5 × 10³ ascospores g⁻¹ soil respectively). The experiment was carried out in 8 replicates. Plants were grown as described above. Plants were harvested when completely dead, and the amount of microsclerotia in the shoot was determined as described above.

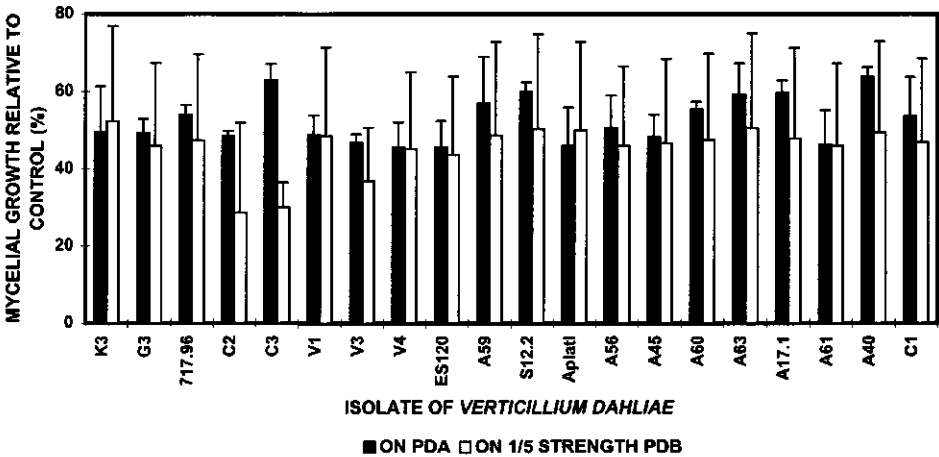


Figure 6.1. Effect of *Pseudomonas fluorescens* strain P60 on mycelial growth of 20 isolates of *Verticillium dahliae* on Potato-Dextrose Agar (PDA) or 1/5 strength Potato-Dextrose Broth amended with 15 g l⁻¹ agar (PDB) relative to growth of *V. dahliae* alone.

Statistical analysis. The area-under-the-senescence-progress curve (AUSPC) was determined according to Campbell & Madden (1990). All experiments were carried out in a completely randomised block design. Data were analysed by using analysis of variance (SPSS for MS Windows Release 6.1), and treatment means were separated by Tukey-HSD test at $P < 0.05$.

Results

Effect of P. fluorescens on V. dahliae in vitro

On PDA, mycelial growth of 20 isolates of *V. dahliae* was reduced by 31-64% when challenged with *P. fluorescens* P60 (Figure 6.1). On 1/5 strength PDB, the inhibition by P60 was slightly less and ranged from 29-52%. Analysis of variance did not indicate a significant difference between *V. dahliae* isolates.

Isolate 717.96 of *V. dahliae* did not produce microsclerotia. All other isolates produced more microsclerotia on PDA than on 1/5 strength PDB agar. *P. fluorescens* P60 reduced formation of microsclerotia on PDA for most isolates of *V. dahliae* (Table 6.2). *V. dahliae* isolates AplatI A60, A63, and A17.1 appeared to be quite sensitive to *P. fluorescens* P60 with

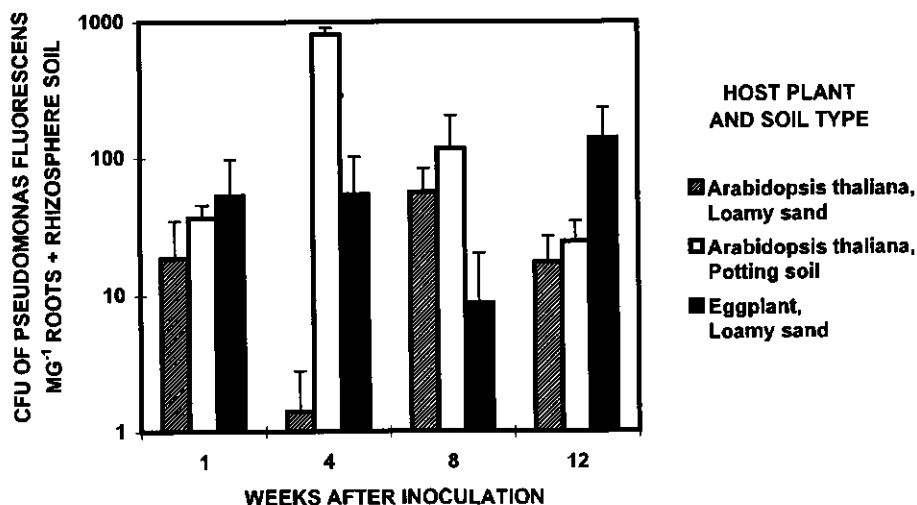


Figure 6.2. Colonization of the rhizosphere of *Arabidopsis thaliana* and eggplant by *Pseudomonas fluorescens* strain P60 at various times after root-dipping with 10^7 cells ml^{-1} of 4- or 3-week-old seedlings respectively.

respect to the formation of microsclerotia, whilst isolates K3, ES120, C2, V3, and V4 were relatively insensitive (Table 6.2). On 1/5 strength PDB agar, control plates showed no or very few microsclerotia (average index 0.85); when challenged with *P. fluorescens* P60, microsclerotia were not formed at all. Similar data were obtained for two other *Pseudomonas* isolates (data not shown). For the plant assays, P60 was chosen because it gave more reduction on formation of microsclerotia of *V. dahliae* *in vitro* than the other strains.

Table 6.2. Effect of *Pseudomonas fluorescens* P60 on the formation of microsclerotia of *Verticillium dahliae* on Potato-Dextrose Agar.

<i>V. dahliae</i> isolate	Control	<i>P. fluorescens</i> P60
K3	2.0 ± 0.0 ¹	1.3 ± 1.5
G3	3.0 ± 0.0	1.3 ± 0.6
717.96	0.0 ± 0.0	0.0 ± 0.0
C2	2.0 ± 0.0	1.0 ± 0.0
C3	1.7 ± 0.6	0.3 ± 0.6
V1	2.7 ± 0.6	1.7 ± 0.6
V3	2.3 ± 0.6	1.3 ± 0.6
V4	2.7 ± 0.6	1.3 ± 0.6
ES120	1.0 ± 0.0	1.3 ± 0.6
A59	2.7 ± 0.6	1.7 ± 0.6
S12.2	1.7 ± 0.6	0.0 ± 0.0
AplatI	2.0 ± 0.0	0.7 ± 0.6
A56	2.0 ± 0.0	0.7 ± 0.6
A45	2.3 ± 0.6	1.0 ± 1.0
A60	2.3 ± 0.6	0.3 ± 0.6
A63	2.7 ± 0.6	0.7 ± 0.6
A17.1	3.0 ± 0.0	1.0 ± 1.0
A61	1.3 ± 0.6	0.7 ± 0.6
A40	1.7 ± 0.6	0.7 ± 0.6
C1	1.7 ± 0.6	0.7 ± 0.6
Average	2.0 ± 0.4	0.9 ± 0.6

¹0 = no microsclerotia, 1 = microsclerotia covering < 25% of the colony, 2 = 25-75%, 3 = > 75%. Values indicated are averages ± S.D. of 3 replicates.

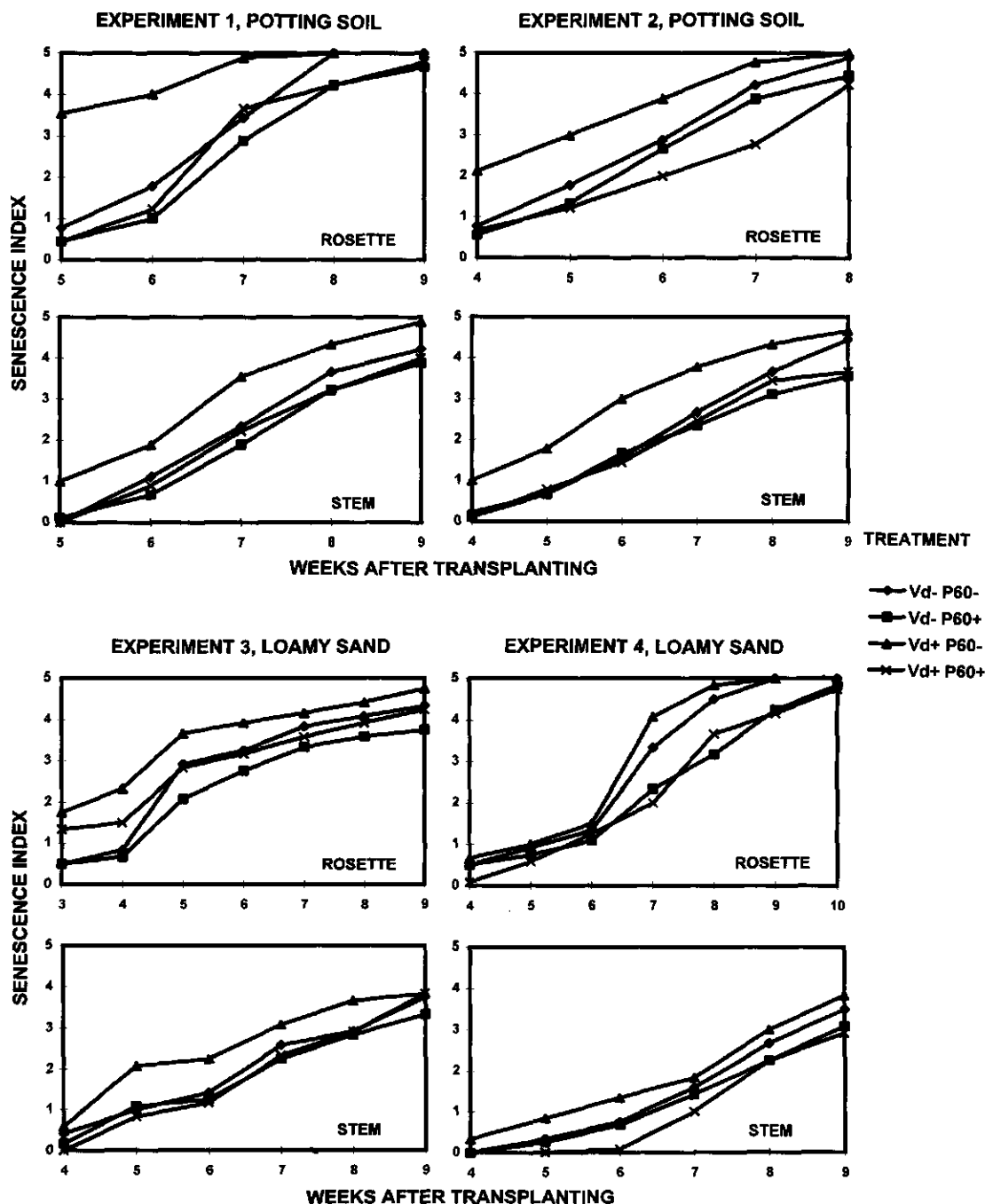


Figure 6.3. Senescence progress of rosette leaves and stems of *Arabidopsis thaliana*, root-dipped or not with 10^7 cells ml^{-1} of *Pseudomonas fluorescens* strain P60, on two soil types infested with 0 or 50 microsclerotia g^{-1} soil of *Verticillium dahliae* isolate A17.1. Senescence Index (from 0 = healthy to 5 = dead) is explained in detail in Materials and Methods.

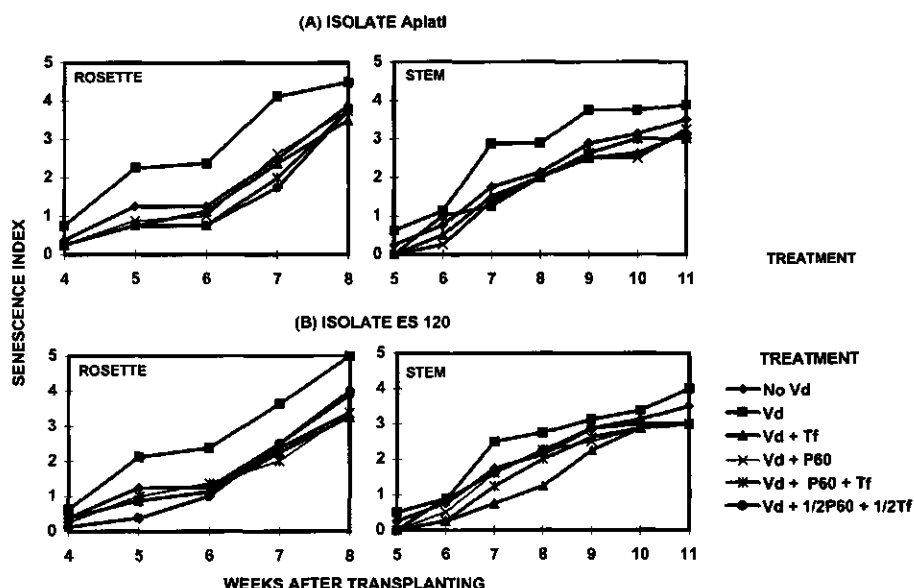


Figure 6.4. Progress of senescence in rosette leaves and stems of *Arabidopsis thaliana*, root-dipped or not with 10^7 cells ml^{-1} of *Pseudomonas fluorescens* strain P60 infested with 0 or 50 microsclerotia g^{-1} soil of *Verticillium dahliae* isolate AplatI (A) or ES120 (B), and with 0 or 10^4 ascospores g^{-1} soil of *Talaromyces flavus* isolate R1. Senescence Index (0 = healthy, 5 = dead) is explained in detail in Materials and Methods.

Colonization of the rhizosphere by *P. fluorescens*

P. fluorescens P60 was able to colonize the rhizosphere of *A. thaliana* and eggplant; it could not be isolated from the interior of the root tissue. Numbers of colony-forming units in the rhizosphere ranged from 1.4×10^3 to 8×10^5 cfu g^{-1} roots; no major differences in population densities were observed between the different test plants and soils (Figure 6.2).

Control of verticillium wilt by *P. fluorescens* P60

Development of senescence in *A. thaliana* was significantly accelerated by *V. dahliae*. In both soil types, introduction of P60 nullified the effect of *V. dahliae* resulting in plants that showed senescence rates similar to those observed in treatments without *V. dahliae* (Figure 6.3 and Table 6.3). P60 slightly reduced stem infection in *A. thaliana* by *V. dahliae* in 3 out of 4 experiments (up to 25%). More importantly, the amount of microsclerotia produced in the shoot decreased 5 to 8-fold upon treatment with P60 (Table 6.3). Infection of the stem bases of eggplant were reduced

by P60 from 27 to 2.1%; root platings showed that the infection was reduced from 83 to 44%. These data were obtained from one experiment only.

Combined application of P. fluorescens and T. flavus

Initially, the *in vitro* assay with P60 showed a slight inhibition of *T. flavus* Tf1 and R1 at 25 and 30°C, but after 4 week of incubation both isolates of *T. flavus* continued to grow, completely covering the bacterial colony. Both P60 and *T. flavus* R1 slowed down senescence of *A. thaliana* to levels that did not differ significantly from the senescence of plants grown in non-infested soil (Figure 6.4). The effects of P60 and *T. flavus* were almost similar and no differences were observed between the level of suppression of the two *V. dahliae* isolates. Application of halved inoculum densities of both antagonists had the same disease-suppressing effect as the full strength inocula. This was confirmed in analysing the area-under-the-senescence-progress curve (Figure 6.5A). P60 and *T. flavus* individually reduced the numbers of microsclerotia approximately 8 and 4-fold respectively. Combination of both biocontrol agents resulted in a 26 and 44 times reduction in the formation of microsclerotia by isolates A17.1 and ES120 respectively. Similarly, halved inoculum densities reduced the number of microsclerotia formed by both isolates of *V. dahliae*, to almost the same level as obtained by full strength inoculum densities of the antagonists (Figure 6.5B).

Discussion

In four bioassays with *A. thaliana* and in two assays with eggplant, *P. fluorescens* strain P60 consistently suppressed verticillium wilt. Suppression by P60 was observed in bioassays performed in both a potting soil and a sandy loam field soil. In these assays, relatively high inoculum densities of *V. dahliae* were used, viz. 50 microsclerotia per gramme of soil. Inoculum densities of 2 to 10 microsclerotia per gramme of soil generally are thought to incite significant damage in susceptible crops (Ashworth *et al.*, 1979; Nicot & Rouse, 1987). Despite the high inoculum density used in this study, the rate of senescence of the shoot and the number of newly formed microsclerotia per unit of shoot dry weight were substantially reduced in *A. thaliana* treated with P60. To our knowledge, this is the first time that a biocontrol agent is shown to substantially lower the number of newly formed microsclerotia of *V. dahliae*. This reduction is likely to affect significantly the disease development in successive croppings of a susceptible host.

P60 colonized the rhizosphere of both *A. thaliana* and eggplant, but could not be isolated

Table 6.3. The effect of *Pseudomonas fluorescens* strain P60 (Pf) on infection, production of new microsclerotia and area-under-the-disease-progress curve (AUSPC), of *Arabidopsis thaliana* growing on soil infested with 0 or 50 microsclerotia g⁻¹ soil of *Verticillium dahliae* isolate A17.1 (Vd) in two soil types.

Treatment	Potting soil										Loamy field sand									
	Experiment 1					Experiment 2					Experiment 3					Experiment 4				
	Pf		AUSPC			Pf		AUSPC			Pf		AUSPC			Pf		AUSPC		
Vd	Inf % ¹	Ms. ²	Rosette	Stem	Inf. %	Ms.	Rosette	Stem	Inf. %	Ms.	Rosette	Stem	Inf. %	Ms.	Rosette	Stem	Inf. %	Ms.	Rosette	Stem
No	0	0	13.2 a ³	9.2 a	0	0	11.2 a	10.9 a	0	0	17.4 a	10.1 a	0	0	12.7 a	11.1 a	0	0	9.7 b	9.7 ab
Yes	0	0	10.8 a	7.8 a	0	0	10.3 a	9.5 a	0	0	16.2 a	9.1 a	0	0	16.60 a	15.8 c	0	0	16.9 c	15.8 c
No	100	4664 a	17.6 b	12.7 b	100	8205 a	15.1 b	15.9 b	100	3549 a	22.1 b	13.9 b	100	639 b	17.8 a	9.2 a	100	384 b	9.9 ab	8.0 a
Yes	89	878 b	11.7 a	8.6 a	89	1083 b	8.2 a	9.5 a	100	639 b	17.8 a	9.2 a	100	639 b	17.8 a	9.2 a	100	384 b	9.9 ab	8.0 a

¹ Stem infection percentage by observation of microsclerotia in senescent shoot tissue.

² Number of microsclerotia g⁻¹ shoot dry weight; plants that did not contain microsclerotia in the shoots were excluded from the data set.

³ Values in each column followed by the same letter are not significantly different according to the Tukey-HSD test ($P < 0.05$).

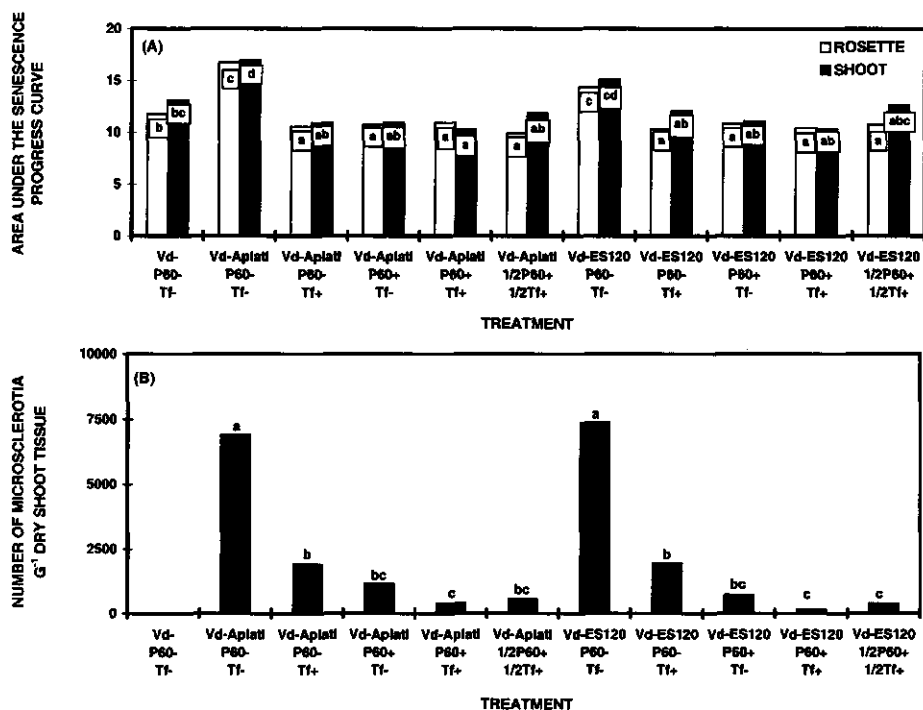


Figure 6.5. Effect of *Pseudomonas fluorescens* strain P60 and *Talaromyces flavus* isolate R1 on (A) area-under-the-disease-progress curve of *Arabidopsis thaliana* growing on soil infested with 0 or 50 microsclerotia g^{-1} soil of *Verticillium dahliae* isolates AplatI or ES120 and (B) the number of microsclerotia g^{-1} dry shoot tissue (shown are back-transformed averages after square root transformation). Bars labelled with the same letter are not significantly different according to the Tukey-HSD test ($P < 0.05$).

from the interior of root or shoot. The adverse effects on *V. dahliae* may be the result of different or multiple mechanisms. A first mechanism may be antibiosis. Preliminary results have shown that strain P60 produces the antibiotic 2,4-diacetylphloroglucinol (DAPG) (Souza and Raaijmakers, unpublished data) which was shown to have profound effects on both mycelial growth and formation of microsclerotia by *V. dahliae* (Soesanto, Raaijmakers and Termorshuizen, unpublished data). Consequently, DAPG production by P60 in the rhizosphere of *A. thaliana* and eggplant may have reduced the number of root infections and the level of colonization of the vascular tissue by *V. dahliae*. The role of DAPG in the interaction between *P. fluorescens* P60 and *V. dahliae* is currently under investigation using purified DAPG and mutants of P60 which are defective in the production of DAPG.

A second mechanism may be related to induced systemic resistance by *P. fluorescens* P60. Induced systemic resistance (ISR) is a widely recognized phenomenon by which root-colonizing fluorescent *Pseudomonas* spp. can suppress plant pathogens (Höfte *et al.*, 1991; Pieterse *et al.*, 1996; Kloepper *et al.*, 1997; Van Loon, 1997). Bioassays (*e.g.* split-root systems), in which the pathogen and strain P60 are physically separated will be required to evaluate the role played by ISR in the control of verticillium wilt by P60 and particularly in the reduction of the number of newly formed microsclerotia. A third mechanism may deal with siderophore-mediated competition for Fe^{III+} in the rhizosphere (Bakker *et al.*, 1993; Duijff *et al.*, 1993).

Joint application of *P. fluorescens* P60 with *T. flavus* reduced the senescence rate of *A. thaliana* to a level found in plants grown in non-infested soil. The production of microsclerotia per unit of shoot dry weight was significantly reduced up to 44 times in the combined applications; the combination was more effective than application of each antagonist separately. Nagtzaam *et al.* (1998) reported that combinations of *T. flavus* with *Bacillus subtilis*, *Fusarium oxysporum*, or *Gliocladium roseum* reduced root colonization and stem infection by *V. dahliae* to a similar extent as application of the single antagonists at rates. Although disease control was not improved, they concluded that combined applications could make biocontrol more consistent under a range of environmental conditions. Antagonist mixtures also have been used in other host-pathogen systems to improve the level of suppression (Fuchs and Défago, 1991; Lemanceau *et al.* 1992; Park *et al.*, 1988). These and other studies show that mixtures can lead to synergistic, additive, or neutral effects on the level of disease suppression. According to Baker (1990), compatibility between different antagonistic strains or agents is an important aspect of successful application of antagonist mixtures. The results of this study suggest that *P. fluorescens* P60 and *T. flavus* are compatible, given the improvement in the reduction of newly formed microsclerotia. Moreover, *in vitro* studies indicate that mycelial growth of *T. flavus* was not adversely affected by P60. We so far have no direct evidence if the two organisms adversely affect each other in the rhizosphere. Possibly both antagonists prefer different microhabitats in the rhizosphere and the joint effect of DAPG produced by *P. fluorescens* P60 and either of glucose oxidase produced or mycoparasitism by *T. flavus* synergistically affect the development of *V. dahliae*. These aspects are currently under investigation.

CHAPTER 7

GENERAL DISCUSSION

A goal of the research described in this thesis was to obtain ecological insight into the dynamics of microsclerotia of *Verticillium dahliae*. Attention was paid to the formation and survival of microsclerotia. Detailed knowledge about factors influencing these processes may lead to new prospects in management of verticillium wilt. Next, possible improvements in the biological control of verticillium wilt were attempted. The idea was confirmed that the above-ground application of the biocontrol agent *Talaromyces flavus* onto microsclerotia-containing debris would lead to reduction of inoculum density of *V. dahliae*. In addition, the effects of a diacetylphloroglucinol-producing strain of *Pseudomonas fluorescens* on verticillium wilt was studied.

Arabidopsis thaliana as a bioassay tool to study the ecology and control of *V. dahliae*

By planting a susceptible, quickly reacting host on soil infested with *V. dahliae*, the ability of *V. dahliae* to infect a host and incite disease was tested directly. Bioassays yield qualitatively different information from that obtained by plating methods, which aim to quantify the microsclerotia in soil. Plating methods are usually considered relatively straightforward in their interpretation (number of microsclerotia per unit of weight or volume of soil; but see discussion in the next paragraph), but bioassays integrate information on all factors leading to infection of *V. dahliae*. An important assumption in performing such bioassays is that the test plants themselves do not influence the soil environment. Therefore, regular checks as to whether the test plant responds in a similar way to the relevant agricultural crop are necessary. In the present study such a check was performed, where suppression of verticillium wilt was obtained both in *A. thaliana* and in eggplant (Chapter 6).

Bioassays have the advantage that some well-known problems associated with plating methods are circumvented (Termorshuizen *et al.*, 1998). However, it should be appreciated that bioassays are not free of problems. The large variation in response to different microsclerotial densities makes it difficult to draw any reliable conclusion about the numbers of microsclerotia in the soil. Therefore, we recommend use mainly for comparative experiments and for detecting very low numbers of microsclerotia in soil that otherwise may escape detection.

Arabidopsis thaliana was chosen as a bioassay plant for *V. dahliae* (Chapter 2) because of its short life cycle. Although crucifers seem to be more frequently infected by *V. longisporum* than by *V. dahliae* in the field (Okoli *et al.*, 1994; Subbarao *et al.*, 1995; Karapapa *et al.*, 1997), under experimental conditions *A. thaliana* appears to be highly susceptible to *V. dahliae*. Conversely, in non-crucifers verticillium wilt is always found to be associated with *V. dahliae*, and not *V. longisporum* (Okoli *et al.*, 1994; Subbarao *et al.*, 1995). However, when either *V. dahliae* or *V. longisporum* is inoculated on crucifer or non-crucifer hosts, respectively, disease regularly develops (Subbarao *et al.*, 1995; Tabrett *et al.*, 1995; Karapapa *et al.*, 1997). Apparently, other mechanisms are responsible for suppressing disease development or dispersal of the pathogen in the field in the incompatible combinations. More research is needed on the effect of *V. dahliae* isolated from a range of different hosts as Bhat and Subbarao (1999) found effects of host origin of *V. dahliae* isolates on verticillium wilt severity across a range of plant species.

A. thaliana was readily infected by different isolates of *V. dahliae* from potato. Disease symptoms included early senescence but no typical verticillium wilt symptoms. Intensity of infection of roots and shoots was correlated with densities of 1, 3, 10, 30, and 100 microsclerotia g⁻¹ soil. A log-linear relation between inoculum density and the area-under-the-disease progress curve was established. Even at the lowest inoculum density of 1 microsclerotium g⁻¹ soil, 5% of the root length was infected with *V. dahliae*, and 30% of the stems. The most sensitive parameter was the number of new microsclerotia formed in the shoot g⁻¹ shoot dry weight. The higher the inoculum density the greater the density of microsclerotia in the shoot. It is concluded that multiple root infections lead to a more intensive colonization of *V. dahliae*. Disease progress was strongest at 20°C, and less drastic at 10, 15, and 25°C. The bioassay can optimally be used for assessing whether or not a field soil is infested with *V. dahliae*. We do not know a more sensitive bioassay test plant for this pathogen. An additional advantage of using *A. thaliana* is the possibility of using genetically homogeneous material.

Factors affecting formation, recovery, and survival of microsclerotia of *V. dahliae*

Microsclerotia are the survival structures of *V. dahliae* that supply the inoculum for subsequent crops. They are formed in large numbers on all plant parts, but particularly in shoot tissue. We showed that the numbers of microsclerotia in shoot tissue are affected by the density of microsclerotia in soil (Chapter 2) and temperature (Chapter 3). In addition, presence of antagonists also affects the formation of microsclerotia in the shoot as shown for *Pseudomonas fluorescens* and *Talaromyces flavus* (see next paragraph).

Temperature appears to play a significant role in multiplication of *V. dahliae* (Chapter 3). The optimum temperature for microsclerotium production is slightly lower than that of mycelial growth in pure culture, but between 15 and 25°C high numbers are generally produced. At low temperatures (5-10°C), the development of *V. dahliae* in plant tissue is apparently slower than the development of the host. When first grown at 20°C and moved to different temperatures at the onset of senescence, the formation of microsclerotia was much higher than for plants that were grown at temperatures below 20°C continuously. The results of the temperature experiments are difficult to extrapolate for the field situation, where diurnal and seasonal fluctuations of temperature prevail. Soil moisture content and air humidity also affect colonization by *V. dahliae* and the response of the host to colonization. The large variation observed in the temperature experiments indicates that certain variables may not have been constant. The formation of microsclerotia depends on a cascade of events: root infection, vascular infection, shoot infection, host effects on the pathogen, and pathogen effects on host growth and senescence. The number of shoot infections also depends on root size. Small differences at the onset of the cascade may result in large differences at the end. Therefore further studies of physiological interactions between host and pathogen are needed.

Wilhelm (1955) found that microsclerotia are able to survive for up to 14 years in soil. However, this statement does not provide information about survival rates during this time. Quantitative data on the survival of microsclerotia are remarkably scarce in the literature. The fact that a rotation of a host with 1 in 4 non-host crops results in yields of the host that are almost equal to yields obtained on soils where a host of *V. dahliae* had never been grown (Bollen *et al.*, 1989), indicates that a considerable portion of the microsclerotia can be inactivated within a few years. Therefore, we studied the dynamics of microsclerotia during the first 1-2 years in the absence of a host (Chapter 4). We repeatedly observed low recoveries one day after incorporation of inoculum into a soil, followed by a delayed increase in recovery. Recoveries reported by Wheeler and Rowe (1995) for a single soil sample fluctuated more strongly with time. A hypothesis was put forward that may explain the differences in recoveries observed. An unanswered, crucial question is whether the phenomenon is also operative in the germination behaviour of microsclerotia in the rhizosphere, or whether it only appears in the agar plates. This could be tested by correlating recovery rate and the number of root infections in a bioassay plant. If variation in numbers of root infections keeps pace with variation in recovery, this might indicate a temporary non-germinability of microsclerotia in the soil. If this is the case, there may be scope for selecting antagonists that affect germination of microsclerotia. Like the observations by Wheeler and Rowe (1995) and Termorshuizen *et al.* (1998), our results indicate that even in one soil sample within relatively short time intervals of a few months large fluctuations can

occur. It should be appreciated that fluctuations in recovery can easily be confused with effects of certain treatments.

We obtained indirect evidence that low recoveries can obscure effects on survival. In Chapter 5, it was found that incorporating 0.25 g potato stems containing microsclerotia resulted in production of about 1200 microsclerotia g⁻¹ soil (applied without *T. flavus*), after 4 and 10 months of incubation (Table 5.3, p. 63). This amount is remarkably high, and it may be questioned whether the application was realistic. From unpublished field data (M.P.M. Nagtzaam, pers. comm.) a dry weight production of 616 g m⁻² shoot tissue of potato plants was determined. Assuming that this tissue contained 1×10^6 microsclerotia per gramme shoot tissue, as was often the case for the material collected, an amount of 6.16×10^8 microsclerotia would have been incorporated in the top-soil layer of 20 cm of 1 m², equalling 3,080 microsclerotia cm⁻³ or 2,369 microsclerotia g⁻¹ soil (bulk soil density = 1.3 g cm⁻³). However, densities exceeding 100 microsclerotia g⁻¹ soil are rarely observed in the field. This can be ascribed to clumping of microsclerotia in host tissue, so that shortly after incorporation of *V. dahliae*-infected organic debris in soil not single microsclerotia, but rather clumps of microsclerotia are assessed. In the experiment where we measured 1,200 microsclerotia g⁻¹ soil, the sample was ground before plating, ensuring that discrete microsclerotia, and not clumps of microsclerotia were counted. In the field the host tissue decomposes and the microsclerotia become gradually segregated; some may germinate in the rhizosphere of a following crop, or die. An increase in inoculum density in time due to the 'segregation' of clumps of microsclerotia could go hand in hand with a decrease due to germination and death. Based on our results, an alternative hypothesis for the low numbers of microsclerotia usually measured in spring is the low recovery due to high numbers of micro-organisms residing on the surface of microsclerotia.

Biocontrol of *V. dahliae* with *Pseudomonas fluorescens* and *Talaromyces flavus*

Both antagonists investigated had remarkably strong effects on *V. dahliae*. The particular strain of *P. fluorescens* used in this study has not been tested before against pathogens other than the causal agent of take-all, *Gaeumannomyces graminis* (Raaijmakers *et al.*, 1997). Phloroglucinol-producing strains of *P. fluorescens* contribute to the decline of *G. graminis* in cereals (Raaijmakers and Weller, 1998). Population densities increase in soils where take-all occurs, but apparently not in soils where other pathogens occur. Nevertheless, phloroglucinol-producing strains of *P. fluorescens* are apparently able to persist in the rhizosphere of dicotyledonous plants and to affect pathogens other than *G. graminis*.

The mechanisms involved in this phenomenon are not yet understood. *P. fluorescens* appeared to clearly depress the formation of microsclerotia by *V. dahliae* *in vitro* and the antibiotic phloroglucinol can be made responsible for this, since similar effects were seen when *V. dahliae* was exposed to the pure compound (own preliminary observations). However, to explain the effects of *P. fluorescens* (and also *Talaromyces flavus*) on the numbers of microsclerotia in shoot tissue of *A. thaliana*, it must be assumed that the antagonists infect the shoot tissue, or that their antibiotics (2,4-diacetylphloroglucinol and talaron respectively) are taken up by the plant. *P. fluorescens* could not be isolated from within plant tissue (Chapter 6), and infection of plant tissue by *T. flavus* has never been reported. Therefore, the possibility of uptake of phloroglucinol and talaron by the plant needs to be studied. It seems however more likely that both antagonists inhibit the germination of microsclerotia, and consequently reduce the number of root infections by *V. dahliae*. As shown in Chapter 2, density of microsclerotia in soil is directly related to numbers of root infections and numbers of microsclerotia in shoot tissue. Induced systemic resistance or direct inactivation of microsclerotia are other possible mechanisms. A direct inactivation of microsclerotia as shown for *T. flavus* in Chapter 5, need not be the only operative mechanism.

There clearly is scope for the application of antagonists to control *V. dahliae*. Low temperatures appear to be less inhibitory to the application of *T. flavus* than thought earlier (Chapter 5). An extensive screening under the prevailing climatic and edaphic conditions in the Netherlands of antagonists may lead to even more efficient strains. Such screenings against verticillium wilt have never been performed in the Netherlands for *T. flavus* nor for *P. fluorescens*.

REFERENCES

- Adams, P.B., 1990. The potential of mycoparasites for biological control of plant diseases. *Annual Review of Phytopathology* 28:59-72.
- Allen, S.J., 1994. The effects of various cultural practices on the epidemiology of *Verticillium* wilt of cotton in New South Wales, Australia. *Book of Abstracts of the 6th International Verticillium Symposium, Dead Sea, Israel. Phytoparasitica* 23:51.
- Al-Rawahi, A.K., and J.G. Hancock, 1998. Parasitism and biological control of *Verticillium dahliae* by *Pythium oligandrum*. *Plant Disease* 92:1100-1106.
- Ashworth, L.J., O.D. McCutcheon, and A.G. George, 1972. *Verticillium albo-atrum*: the quantitative relationship between inoculum density and infection of cotton. *Phytopathology* 62:901-903.
- Ashworth, L.J., O.C. Huisman, R.G. Grogan, and D.M. Harper, 1976. Copper induced fungistasis of microsclerotia of *Verticillium albo-atrum* and its influence on infection of cotton in the field. *Phytopathology* 66: 970-977.
- Ashworth, L.J., O.C. Huisman, D.M. Harper, L.K. Stromberg, and D.M. Bassett, 1979. Verticillium wilt disease of cotton: influence of inoculum density in the field. *Phytopathology* 69:483:489.
- Atkinson, T.G., 1981. Verticillium wilt of alfalfa: challenge and opportunity. *Canadian Journal of Plant Pathology* 3:266-272.
- Baker, K.F., and R.J. Cook, 1974. *Biological control of plant pathogens*. Freeman, San Francisco.
- Baker, R., 1990. An overview of current and future strategies and models for biological control. Pp. 375-389. In: D. Hornby (ed.), *Biological control of soil-borne plant pathogens*. C.A.B. International, Wallingford.
- Bakker, P.A.H.M., J.M. Raaijmakers, and B. Schippers, 1993. Role of iron in the suppression of bacterial plant pathogens by fluorescent pseudomonads. Pp. 269-278. In: L.L. Barton and B.C. Hemming (ed.), *Iron chelation in plants and soil microorganisms*. Academic Press, San Diego.
- Bejarano-Alcázar, J., J.M. Melero-Vara, M.A. Blanco-López, and R.M. Jiménez-Díaz, 1995. Influence of inoculum density of defoliating and non-defoliating pathotypes of *Verticillium dahliae* on epidemics of verticillium wilt of cotton in southern Spain. *Phytopathology* 85:1474-1481.
- Bejarano-Alcázar, J., M.A. Blanco-López, J.M. Melero-Vara, and R.M. Jiménez-Díaz, 1996. Etiology, importance, and distribution of verticillium wilt of cotton in southern Spain. *Plant Disease* 80:1233-1238.
- Bejarano-Alcázar, J., A.J. Termorshuizen, and R.M. Jiménez-Díaz, 1999. Single-site root inoculations on eggplant with microsclerotia of *Verticillium dahliae*. *Phytoparasitica* 27:279-289.
- Bell, A.A., 1992. Verticillium wilt. Pp. 87-126. In: R.J. Hillocks (ed.), *Cotton diseases*. C.A.B. International, Wallingford, UK.
- Ben-Yephet, Y., and Y. Pinkas, 1977. Germination of individual microsclerotia of *Verticillium dahliae*. *Phytoparasitica* 5:159-166.
- Berg, G., 1997. Bacterial antagonists to *Verticillium dahliae* var. *longisporum* Stark in the rhizosphere of oilseed rape. *Book of Abstracts of the 7th International Verticillium Symposium*, Athens, Greece, p. 64.
- Berg, G., and G. Ballin, 1994. Bacterial antagonists to *Verticillium dahliae* Kleb. *Journal of Phytopathology* 141:99-110.
- Berg, G., C. Knaape, G. Ballin, and D. Seidel, 1994. Biological control of *Verticillium dahliae* Kleb. by naturally occurring rhizosphere bacteria. *Archive of Phytopathology and Plant Protection* 29:249-262.
- Bhat, R.G., and K.V. Subbarao, 1999. Host range specificity in *Verticillium dahliae*. *Phytopathology* 89:1218-1225.
- Bin, L., G.R. Knudsen, and D.J. Eschen, 1991. Influence of an antagonistic strain of *Pseudomonas fluorescens* on growth and ability of *Trichoderma harzianum* to colonize sclerotia of *Sclerotinia sclerotiorum* in soil. *Phytopathology* 81:994-1000.

- Blanco-López, M.A., R.M. Jiménez Díaz, and J.M. Caballero, 1984. Symptomatology, incidence and distribution of *Verticillium* wilt of olive trees in Andalucía. *Phytopathologia Mediterranea* 23:1-8.
- Blok, W.J., J.G. Lamers, A.J. Termorshuizen, and G.J. Bollen, 2000. Control of soilborne plant pathogens by incorporating fresh organic amendments followed by tarping. *Phytopathology* 90 (in press).
- Bollen, G.J., O. Hoekstra, K. Scholte, T.W. Hofman, M.J. Celetti, and A. Schirring, 1989. Incidence of soilborne pathogens in potato related to the frequency of potato growing on a clay loam. Pp. 203-222. In: J. Vos, C.D. van Loon, and G.J. Bollen (eds.), *Effects of crop rotation on potato production in the temperate zones*. Kluwer Scientific Publishers, Dordrecht.
- Boosalis, M.G., 1956. Effect of soil temperature and green manure amendment of unsterilized soil on parasitism of *Rhizoctonia solani* by *Penicillium vermiculatum* and *Trichoderma* sp. *Phytopathology* 46:473-478.
- Bowden, R.L., and D.I. Rouse, 1991. Effects of *Verticillium dahliae* on gas exchange of potato. *Phytopathology* 81:293-301.
- Bowen, G.D., and A.D. Rovira, 1976. Microbial colonization of plant roots. *Annual Review of Phytopathology* 14:121-144.
- Bowers, J.H., S.T. Nameth, R.M. Riedel, and R.C. Rowe, 1996. Infection and colonization of potato roots by *Verticillium dahliae* as affected by *Pratylenchus penetrans* and *P. crenatus*. *Phytopathology* 86:614-621.
- Brinkerhoff, L.A., 1969. The influence of temperature, aeration, and soil microflora on microsclerotial development of *Verticillium albo-atrum* in abscised cotton leaves. *Phytopathology* 59:805-808.
- Bristow, P.R., and J.L. Lockwood, 1975. Soil fungistasis: role of spore exudates in the inhibition of nutrient-independent propagules. *Journal of General Microbiology* 90:140-146.
- Campbell, C.L., and L.V. Madden, 1990. *Introduction to plant disease epidemiology*. John Wiley & Sons, New York.
- Church, V.J., and H.A. McCartney, 1995. Occurrence of *Verticillium dahliae* on sunflower (*Helianthus annuus*) in the UK. *Annals of Applied Biology* 127:49-56.
- Cirulli, M., F. Ciccarese, and M. Amenduni, 1994. Evaluation of Italian clones of artichoke for resistance to *Verticillium dahliae*. *Plant Disease* 78:680-682.
- Conn, K.L., and G. Lazarovits, 1998. Impact of animal manures on verticillium wilt, potato scab, and soil microbial populations. *Canadian Journal of Plant Pathology* 21:81-92.
- Cook, R.J., 1973. Influence of low plant and soil water potential on diseases caused by soilborne fungi. *Phytopathology* 63:451-458.
- Daebeler, F., D. Amelung, and K. Zeise, 1988. Verticillium wilt on winter rape occurrence and importance. *Nachrichtenblatt für den Pflanzenschutz in der DDR* 42:71-73.
- Davis, J.R., and D.O. Everson, 1986. Relation of *Verticillium dahliae* in soil and potato tissue, irrigation method, and N-fertility to verticillium wilt of potato. *Phytopathology* 76:730-736.
- Davis, J.R., J.J. Pavek, and D.L. Corsini, 1983. A sensitive method for quantifying *Verticillium dahliae* colonization in plant tissue and evaluating resistance among potato genotypes. *Phytopathology* 73:1009-1014.
- Davis, J.R., O.C. Huisman, D.T. Westermann, S.L. Hafez, D.O. Everson, L.H. Sorensen, and A.T. Schneider, 1996. Effects of green manures on verticillium wilt of potato. *Phytopathology* 86:444-453.
- DeVay, J.E., L.L. Forrester, R.H. Garber, and E.J. Butterfield, 1974. Characteristics and concentration of propagules of *Verticillium dahliae* in air-dried field soils in relation to the prevalence of Verticillium wilt in cotton. *Phytopathology* 64:22-29.
- Domsch, K.H., W. Gams, and T. Anderson, 1980. *Compendium of soil fungi*, vol. 1. Academic Press, London.
- Douglas, V.S., W.D. Gubler, and J. Hansen, 1997. Field resistance of California strawberries to *Verticillium dahliae* at three conidial inoculum concentrations. *HortScience* 32:711-713.

- Duijff, B.J., J.W. Meijer, P.A.H.M. Bakker, and B. Schippers, 1993. Siderophore-mediated competition for iron and induced resistance in the suppression of Fusarium wilt of carnation by fluorescent *Pseudomonas* spp. *Netherlands Journal of Plant Pathology* 99:277-289.
- Dunn, M.T., and R.D. Lumsden, 1981. Fungi associated with *Sclerotinia minor* buried in various soils. *Phytopathology* 71:766.
- Eastburn, D.M., and R.J. Chang, 1994. *Verticillium dahliae*: a causal agent of root discoloration of horseradish in Illinois. *Plant Disease* 78:496-498.
- Emmatty, D.A., and R.J. Green, 1969. Fungistasis and the behaviour of the microsclerotia of *Verticillium albo-atrum* in soil. *Phytopathology* 59:1590-1595.
- Evans, G., W.C. Snyder, and S. Wilhelm, 1966. Inoculum increase of the verticillium wilt fungus in cotton. *Phytopathology* 56:590-594.
- Evans, G., C.D. McKeen, and A.C. Gleeson, 1974. A quantitative bioassay for determining low numbers of microsclerotia of *Verticillium dahliae* in field soils. *Canadian Journal of Microbiology* 20:119-124.
- Fahima, T., and Y. Henis, 1980. Interactions between pathogen, host, and biocontrol agent: Multiplication of *Trichoderma hamatum* and *Talaromyces flavus* on roots of diseased and healthy hosts. Pp. 165-180. In: D. Hornby (ed.), *Biological control of soilborne plant pathogens*. C.A.B. International, Wallingford.
- Fahima, T., L. Madi, and Y. Henis, 1992. Ultrastructure and germinability of *Verticillium dahliae* microsclerotia parasitized by *Talaromyces flavus* on agar medium and in treated soil. *Biocontrol Science and Technology* 2:69-78.
- Fahima, T., and Y. Henis, 1995. Quantitative assessment of the interaction between the antagonistic fungus *Talaromyces flavus* and the wilt pathogen *Verticillium dahliae* on eggplant roots. *Plant and Soil* 176:129-137.
- Farley, J.D., S. Wilhelm, and W.C. Snyder, 1971. Repeated germination and sporulation of microsclerotia of *Verticillium albo-atrum* in soil. *Phytopathology* 61:260-264.
- Faulkner, L.R., W.J. Bolander, and C.B. Skotland, 1970. Interaction of *Verticillium dahliae* and *Pratylenchus minyus* in verticillium wilt of peppermint: Influence of the nematode as determined by a double root technique. *Phytopathology* 60:100-103.
- Ferrandino, F.J., 1995. How verticillium wilt affects yield of eggplant, tomatoes and potatoes. *Frontiers of Plant Science* 47:2-5.
- Fernández, M.C., and C.G. Tobar, 1989. Identification of *Verticillium dahliae* Kleb. on artichoke (*Cynara scolymus*). *Agricultura Tecnica Santiago* 49:161-163.
- Fitt, B.D.L., J.W. Cook, and S. Burhenne, 1991. Verticillium on linseed (*Linum usitatissimum*) in the United Kingdom. *Annals of Applied Biology* 28:91-94.
- Fitt, B.D.L., F. Bauers, S. Burhenne, and V.H. Paul, 1992. Occurrence of *Verticillium dahliae* on linseed (*Linum usitatissimum*) in the UK and Germany. *Plant Pathology* 41:86-90.
- Fitzell, R., G. Evans, and P.C. Fahy, 1980. Studies on the colonisation of plant roots by *Verticillium dahliae* with use of immunofluorescent staining. *Australian Journal of Botany* 28:357-368.
- Francel, L.J., L.V. Madden, R.C. Rowe, and R.M. Ridel, 1990. Correlation of growing season environmental variables and the effect of early dying on potato yield. *Phytopathology* 80:425-432.
- Fravel, D.R., J.J. Marois, R.D. Lumsden, and W.J. Connick, 1985. Encapsulation of potential biocontrol agents in an alginate-clay matrix. *Phytopathology* 75:774-777.
- Fravel, D.R., J.R. Davis, and L.H. Sorensen, 1986. Effect of *Talaromyces flavus* and metham sodium on Verticillium wilt incidence and potato yield 1984-1985. *Biological and Cultural Tests for Control of Plant Diseases* 1:17.
- Fravel, D.R., 1996. Interaction of biocontrol fungi with sublethal rates of metham sodium for control of *Verticillium dahliae*. *Crop Protection* 15:115-119.

- Friebertshauser, G.E., and J.E. De Vay, 1982. Differential effects of the defoliating and nondefoliating pathotypes of *Verticillium dahliae* upon the growth and development of *Gossypium hirsutum*. *Phytopathology* 72:872-877.
- Fuchs, J., and G. Defago, 1991. Protection of tomatoes against *Fusarium oxysporum* f.sp. *lycopersici* by combining a non-pathogenic *Fusarium* with different bacteria in untreated soil. Pp. 51-56. In: C. Keel, B. Koller, and G. Defago (Eds.), *Plant growth promoting rhizobacteria*. Interlaken, Switzerland.
- Gams, W., 1992. The analysis of communities of saprophytic microfungi with special reference to soil fungi. Pp. 183-223. In: W. Winterhoff (ed.), *Fungi in vegetation science*. Kluwer Academic Publishers, Dordrecht.
- Gaudreault, S.M., M.L. Powelson, N.W. Christensen, and F.J. Crowe, 1995. Soil water pressure and *Verticillium dahliae* interactions on potato. *Phytopathology* 85:1542-1546.
- Georgieva, O., 1992. Antagonistic characteristics of *Trichoderma koningii* towards *Verticillium dahliae* on pepper. *Bulletin OILB SROP* 15:18-20.
- Gerik, J.S., and O.C. Huisman, 1985. Mode of colonization of roots by *Verticillium* and *Fusarium*. Pp. 80-83. In: C.A. Parker, A.D. Rovira, K.J. Moore, and P.T.W. Wong (eds.), *Ecology and management of soilborne plant pathogens*. The American Phytopathological Society, St. Paul, Minnesota, U.S.A.
- Gerik, J.S., and O.C. Huisman, 1988. Study of field-grown cotton roots infected with *Verticillium dahliae* using an immunoenzymatic technique. *Phytopathology* 78:1174-1178.
- Green, R.J., and G.C. Papavizas, 1968. The effect of carbon source, carbon to nitrogen ratios, and organic amendments on survival of propagules of *Verticillium albo-atrum* in soil. *Phytopathology* 58:567-570.
- Green, R.J., 1969. Survival and inoculum potential of conidia and microsclerotia of *Verticillium albo-atrum* in soil. *Phytopathology* 59:874-876.
- Green, R.J., 1980. Soil factors affecting survival of microsclerotia of *Verticillium dahliae*. *Phytopathology* 70:353-355.
- Hall, R., and L.V. Busch, 1971. *Verticillium* wilt of chrysanthemum: colonization of leaves in relation to symptom development. *Canadian Journal of Botany* 49:181-185.
- Harris, D.C., J.R. Yang, and M.S. Ridout, 1993. The detection and estimation of *Verticillium dahliae* in naturally infested soil. *Plant Pathology* 42:238-250.
- Harrison, J.A.C., 1971. Association between the potato cyst nematode *Heterodera rostochiensis* Woll. and *Verticillium dahliae* Kleb. in the early dying disease of potatoes. *Annals of Applied Biology* 67:185-193.
- Harrison, M.D., 1976. The effect of barley straw on the survival of *Verticillium albo-atrum* in naturally infested field soil. *American Potato Journal* 53:385-394.
- Hashimoto, K., 1989. Studies on verticillium wilt of eggplant. *Bulletin of the Saitama Horticultural Experiment Station* 2, 110 pp.
- Haverkort, A.J., D.I. Rouse, and L.J. Turkensteen, 1990. The influence of *Verticillium dahliae* and drought on potato crop growth. 1. Effects on gas exchange and stomatal behaviour of individual leaves and crop canopies. *Netherlands Journal of Plant Pathology* 96:273-289.
- Hawke, M.A., and G. Lazarovits, 1994. Production and manipulation of individual microsclerotia of *Verticillium dahliae* for use in studies of survival. *Phytopathology* 84:883-890.
- Hawke, M.A., and G. Lazarovits, 1995. The role of melanin in the survival of microsclerotia of *Verticillium dahliae*. *Phytoparasitica* 23:54.
- Heale, J.B., and D.P. Gupta, 1972. Mechanism of vascular wilting induced by *Verticillium albo-atrum*. *Transactions of the British mycological Society* 58:19-28.
- Heale, J.B., and I. Isaac, 1965. Environmental factors in the production of dark resting structures in *Verticillium albo-atrum*, *V. dahliae* and *V. tricorpus*. *Transactions of the British mycological Society* 48:39-50.

- Heffer, V.J., and R.P. Regan, 1996. First report of verticillium wilt caused by *Verticillium dahliae* of ash trees in Pacific Northwest nurseries. *Plant Disease* 80:342.
- Hiemstra, J.A., 1998. Some general features of verticillium wilt in trees. Pp. 5-11. In: J.A. Hiemstra, and D.C. Harris (Eds.), *A compendium of verticillium wilt in tree species*. Ponsen & Looijen, Wageningen, the Netherlands.
- Höfte, M., K.Y. Seong, E. Jurkevitch, and W. Verstraete, 1991. Pyoverdinin production by the plant growth beneficial *Pseudomonas* strain 7NSK2: ecological significance in soil. *Plant and Soil* 130:249-257.
- Hsu, S.C., and J.L. Lockwood, 1973. Soil fungistasis: Behaviour of nutrient-independent spores and sclerotia in a model system. *Phytopathology* 63:334-337.
- Huisman, O.C., and L.J. Ashworth, 1976. Influence of crop rotation on survival of *Verticillium albo-atrum* in soils. *Phytopathology* 66:978-981.
- Huisman, O.C., 1982. Interrelations of root growth dynamics to epidemiology of root-invading fungi. *Annual Review of Phytopathology* 20:303-327.
- Huisman, O.C., 1988. Seasonal colonization of roots of field-grown cotton by *Verticillium dahliae* and *V. tricorpus*. *Phytopathology* 78:708-716.
- Huisman, O.C., and J.S. Gerik, 1989. Dynamics of colonization of plant roots by *Verticillium dahliae* and other fungi. Pp. 1-17. In: Tjamos, E.C. and C.H. Beckman (eds.), *Vascular wilt diseases of plants*. Springer Verlag, Berlin.
- Hyakumachi, M., and J.L. Lockwood, 1989. Relation of carbon loss from sclerotia of *Sclerotium rolfsii* during incubation in soil to decreased germinability and pathogenic aggressiveness. *Phytopathology* 79:1059-1063.
- Ioannou, N., R.W. Schneider, R.G. Grogan, and J.M. Duniway, 1977a. Effect of water potential and temperature on growth, sporulation, and production of microsclerotia by *Verticillium dahliae*. *Phytopathology* 67:637-644.
- Ioannou, N., R.W. Schneider, and R.G. Grogan, 1977b. Effect of flooding on the soil gas composition and the production of microsclerotia by *Verticillium dahliae* in the field. *Phytopathology* 67:651-656.
- Isaac, I., 1953. A further comparative study of pathogenic isolates of *Verticillium*: *V. nubilum* Pethybr. and *V. tricorpus* sp. nov. *Transactions of the British mycological Society* 36:180-195.
- Jackson, C.W., and J.B. Heale, 1985. Relationship between DNA content and spore volume in sixteen isolates of *Verticillium lecanii* and two new diploids of *V. dahliae* (= *V. dahliae* var. *longisporum* Stark). *Journal of General Microbiology* 131:3229-3236.
- Jeger, M.J., G.A. Hide, P.H.J.F. van den Boogert, A.J. Termorshuizen, and P. van Baarlen, 1996. Pathology and control of soilborne fungal pathogens of potato. *Potato Research* 39:437-469.
- Jiménez-Díaz, R.M., and R.L. Millar, 1988. Sporulation on infected tissues, and presence of airborne *Verticillium albo-atrum* in alfalfa fields in New York. *Plant Pathology* 37:64-70.
- Joaquim, T.R., and R.C. Rowe, 1991. Vegetative compatibility and virulence of strains of *Verticillium dahliae* from soil and potato plants. *Phytopathology* 81:552-558.
- Johnston, S.A., J. Lashomb, and G.C. Hamilton, 1994. Evaluation of the integration of soil fumigation and microbial antagonists for the control Verticillium wilt of eggplant, 1993. *Biological and Cultural Tests* 9:28.
- Jordan, V.W.L., B. Sneh, and B.P. Eddy, 1972. Influence of organic soil amendments on *Verticillium dahliae* and on the microbial composition of the strawberry rhizosphere. *Annals of Applied Biology* 70:139-148.
- Karapapa, V.K., B.W. Bainbridge, and J.B. Heale, 1997. Morphological and molecular characterization of *Verticillium longisporum* comb. nov., pathogenic to oilseed rape. *Mycological Research* 101:1281-1294.
- Katan, J., A. Greenberger, A. Alon, and A. Grinstein, 1976. Solar heating by polyethylene mulching for the control of diseases caused by soil-borne plant pathogens. *Phytopathology* 66:683-688.
- Katan, J., 1980. Solar pasteurization of soils for disease control: Status and prospects. *Plant Disease* 64:450-454.

- Katan, J., 1981. Solar heating (solarization) of soil for control of soilborne pests. *Annual Review of Phytopathology* 19:211-236.
- Keinath, A.P., and D.R. Fravel, 1992. Induction of soil suppressiveness to verticillium wilt of potato by successive croppings. *American Potato Journal* 69:503-513.
- Keinath, A.P., D.R. Fravel, and G.C. Papavizas, 1991. Potential of *Gliocladium roseum* for biocontrol of *Verticillium dahliae*. *Phytopathology* 81:644-648.
- Kersten, H., 1997. Formulation of a fungicide, based on ascospores of *Talaromyces flavus* to control *Verticillium dahliae*. *Book of Abstracts of the 7th International Verticillium Symposium*, Athens, Greece, p. 67.
- Klisiewicz, J.M., 1975. Survival and dissemination of *Verticillium* in infected safflower seed. *Phytopathology* 65:696-698.
- Kloepper, J.W., S. Tuzun, G.W. Zehnder, and G. Wei, 1997. Multiple disease protection by rhizobacteria that induce systemic resistance—historical precedence. *Phytopathology* 136:137.
- Koch, E., 1999. Evaluation of commercial products for microbial control of soil-borne plant diseases. *Crop Protection* 18:119-125.
- Koch, E., and A.J. Slusarenko, 1990. Fungal pathogens of *Arabidopsis thaliana* (L.) Heyhn. *Botanica Helvetica* 100:257-269.
- Koike, S.T., J.H. Nelson, and D.K. Perry, 1991. Verticillium wilt of protea (*Leucospermum cordifolium*) caused by *Verticillium dahliae*. *Plant Disease* 75:1074.
- Koike, S.T., and K.V. Subbarao, 1995. Verticillium wilt of cauliflower: a new disease in California. *Phytoparasitica* 23:52.
- Koike, S.T., K.V. Subbarao, R.M. Davis, T.R. Gordon, and J.C. Hubbard, 1994. Verticillium wilt of cauliflower in California. *Plant Disease* 78:1116-1121.
- Koike, S.T., C.L. Xiao, J.C. Hubard, K.F. Schulbach, and K.V. Subbarao, 1997. Effects of broccoli residue on the cauliflower-*Verticillium dahliae* host. *Book of Abstracts of the 7th International Verticillium Symposium*, Athens, Greece, p. 80.
- Korolev, N., and T. Katan, 1999. Vegetative compatibility grouping in *Verticillium nigrescens* and *V. tricorpus*. *Mycological Research* 103:65-76.
- Lacy, M.L., and C.E. Horner, 1966. Behaviour of *Verticillium dahliae* in the rhizosphere and on roots of plants susceptible, resistant, and immune to wilt. *Phytopathology* 56:427-430.
- Latorre, B.A., and P.T. Allende, 1983. Occurrence and incidence of Verticillium wilt in Chilean avocado groves. *Plant Disease* 67:445-447.
- Lazarovits, G., M.A. Hawke, Th.H.A. Olthof, and J. Coutu-Sundy, 1991. Influence of temperature on survival of *Pratylenchus penetrans* and of microsclerotia of *Verticillium dahliae* in soil. *Canadian Journal of Plant Pathology* 13:106-111.
- Leben, S.D., J.A. Wadi, and G.D. Easton, 1987. Effects of *Pseudomonas fluorescens* on potato plant growth and control of *Verticillium dahliae*. *Phytopathology* 77:1592-1595.
- Lemanceau, P., and C. Alabouvette, 1991. Biological control of fusarium diseases by fluorescent *Pseudomonas* and non-pathogenic *Fusarium*. *Crop Protection* 10:279-286.
- Lemanceau, P., P.A.H.M. Bakker, W.J. de Kogel, C. Alabouvette, and B. Schippers, 1992. Effect of pseudobactin 358 production by *Pseudomonas putida* WCS358 on suppression of fusarium wilt of carnations by nonpathogenic *Fusarium oxysporum* Fo47. *Applied and Environmental Microbiology* 58:2978-2982.
- Lewis, J.A., and G.C. Papavizas, 1985. Characteristics of alginate pellets formulated with *Trichoderma* and *Gliocladium* and their effect on the proliferation of the fungi in soil. *Plant Pathology* 34:571-577.
- Lockwood, J.L., 1977. Fungistasis in soils. *Biological Reviews* 52:1-43.
- Luisi, N., F. Ciccarese, G. Sicoli, M. Amenduni, and G. Barbera, 1994. Outbreaks of verticillium wilt on almond and pathogenic variations among isolates of *V. dahliae*. *Acta Horticulturae* 373:287-292.
- Luisi, N., and G. Sicoli, 1993. Severe dieback of almond trees in Italy caused by *Verticillium dahliae* Kleb. *Informatore Fitopatologico* 43:45-48.

- Lynch, J.M., and J.M. Whipps, 1990. Substrate flow in the rhizosphere. *Plant and Soil* 129:1-10.
- MacGuidwin, A.E., and D.I. Rouse, 1990a. Effect of *Meloidogyne hapla*, alone or in combination with subthreshold populations of *Verticillium dahliae*, on disease symptomatology and yield of potato. *Phytopathology* 80:482-486.
- MacGuidwin, A.E., and D.I. Rouse, 1990b. Role of *Pratylenchus penetrans* in potato early dying disease of Russet Burbank potato. *Phytopathology* 80:1077-1082.
- Madi, L., T. Katan, and Y. Henis, 1992. Inheritance of antagonistic properties and lytic enzyme activities in sexual crosses of *Talaromyces flavus*. *Annals of Applied Biology* 121:565-576.
- Madi, L., T. Katan, J. Katan, and Y. Henis, 1997. Biological control of *Sclerotium rolfsii* and *Verticillium dahliae* by *Talaromyces flavus* is mediated by different mechanisms. *Phytopathology* 87:1054-1060.
- Mai, W.F., and G.S. Abawi, 1987. Interactions among root-knot nematodes and fusarium wilt fungi on host plants. *Annual Review of Phytopathology* 25:317-338.
- Malik, N.K., and J.M. Milton, 1980. Survival of *Verticillium* in monocotyledonous plants. *Transactions of the British mycological Society* 75:496-498.
- Mansoori, B., J.M. Milton, and C.J. Smith, 1995. Isolation and partial purification of a phytotoxin related to pathogenic *Verticillium* species. *Journal of Phytopathology* 143:33-36.
- Marois, J.J., D.R. Fravel, and G.C. Papavizas, 1984. Ability of *Talaromyces flavus* to occupy the rhizosphere and its interaction with *Verticillium dahliae*. *Soil Biology and Biochemistry* 16:387-390.
- Marois, J.J., S.A. Johnston, M.T. Dunn, and G.C. Papavizas, 1982. Biological control of verticillium wilt of eggplant in the field. *Plant Disease* 66:1166-1168.
- Mathre, D.E., 1986. Occurrence of *Verticillium dahliae* on barley. *Plant Disease* 70:981.
- Mathre, D.E., 1989. Pathogenicity of an isolate of *Verticillium dahliae* from barley. *Plant Disease* 73:164-167.
- McKeen, C.D., and H.J. Thorpe, 1981. Verticillium wilt of potato in southwestern Ontario and survival of *Verticillium albo-atrum* and *V. dahliae* in field soil. *Canadian Journal of Plant Pathology* 3:40-46.
- McLaren, D.L., H.C. Huang, and S.R. Rimmer, 1986. Hyperparasitism of *Sclerotinia sclerotiorum* by *Talaromyces flavus*. *Canadian Journal of Plant Pathology* 8:43-48.
- Melero-Vara, J.M., M.A. Blanco-López, J. Bejarano-Alcázar, and R.M. Jiménez-Díaz, 1995. Control of verticillium wilt of cotton by means of soil solarization and tolerant cultivars in southern Spain. *Plant Pathology* 44:250-260.
- Menzies, J.D., and G.E. Griebel, 1967. Survival and saprophytic growth of *Verticillium dahliae* in uncropped soil. *Phytopathology* 57:703-709.
- Miller, J.F., and T.J. Gulya, 1995. Registration of germplasm. *Crop Science* 35:286.
- Mol, L., 1995a. The influence of plant roots on the germination of microsclerotia of *Verticillium dahliae* in the soil. *Phytoparasitica* 23:54.
- Mol, L., 1995b. Effect of plant roots on the germination of microsclerotia of *Verticillium dahliae*. II. Quantitative analyses of the luring effects of crops. *European Journal of Plant Pathology* 101:679-685.
- Mol, L., 1995c. Formation of microsclerotia of *Verticillium dahliae* on various crops. *Netherlands Journal of Agricultural Science* 43:205-215.
- Mol, L., 1995d. Agronomic studies on the population dynamics of *Verticillium dahliae*. Ph.D. Thesis Wageningen Agricultural University, 159 pp.
- Mol, L., K. Scholte, and J. Vos, 1995. Effects of crop rotation and removal of crop debris on the soil population of two isolates of *Verticillium dahliae*. *Plant Pathology* 44:1070-1074.
- Mol, L., J.M. van Halteren, K. Scholte, and P.C. Struik, 1996a. Effects of crop species, crop cultivars and isolates of *Verticillium dahliae* on the population of microsclerotia in the soil, and consequences for crop yield. *Plant Pathology* 45:205-214.

- Mol, L., O.C. Huisman, K. Scholte, and P.C. Struik, 1996b. Theoretical approach to the dynamics of the inoculum density of *Verticillium dahliae* in the soil: first test of a simple model. *Plant Pathology* 45:192-204.
- Mol, L., and H.W. van Riesen, 1995. Effect of plant roots on the germination of microsclerotia of *Verticillium dahliae*. I. Use of root observation boxes to assess differences among crops. *European Journal of Plant Pathology* 101:673-678.
- Mol, L., and K. Scholte, 1995a. Formation of microsclerotia of *Verticillium dahliae* Kleb. on various plant parts of two potato cultivars. *Potato Research* 38:143-150.
- Mol, L., and K. Scholte, 1995b. Effect of haulm treatments on the formation of microsclerotia of *Verticillium dahliae* Kleb. on potato. *Potato Research* 38:151-157.
- Mol, L., K. Scholte, and J. Vos, 1995. Effects of crop rotation and removal of crop debris on the soil population of two isolates of *Verticillium dahliae*. *Plant Pathology* 44:1070-1074.
- Nadakavukaren, M.J., and C.E. Horner, 1959. An alcohol agar medium selective for determining *Verticillium* microsclerotia in soil. *Phytopathology* 49:527-528.
- Nagtzaam, M.P.N., 1998. Biological control of *Verticillium dahliae* by *Talaromyces flavus*. PhD Thesis. Wageningen Agricultural University, Wageningen, the Netherlands.
- Nagtzaam, M.P.N., and G.J. Bollen, 1997. Colonization of roots of eggplant and potato by *Talaromyces flavus* from coated seed. *Soil Biology and Biochemistry* 29:1499-1507.
- Nagtzaam, M.P.M., G.J. Bollen, and A.J. Termorshuizen, 1998. Efficacy of *Talaromyces flavus* alone or in combination with other antagonists in controlling *Verticillium dahliae* in growth chamber experiments. *Journal of Phytopathology* 146:165-173.
- Nagtzaam, M.P.M., A.J. Termorshuizen, and G.J. Bollen, 1997. The relationship between soil inoculum density and plant infection as a basis for a quantitative bioassay of *Verticillium dahliae*. *European Journal of Plant Pathology* 103:597-605.
- Nicot, P.C., and D.I. Rouse, 1987. Relationship between soil inoculum density of *Verticillium dahliae* and systemic colonization of potato stems in commercial fields over time. *Phytopathology* 77:1346-1355.
- Okoli, C.A.N., J.H. Carder, and D.J. Barbara, 1994. Restriction fragment length polymorphisms (RFLPs) and the relationships of some host-adapted isolates of *Verticillium dahliae*. *Plant Pathology* 43:33-40.
- Olsson, S., and B. Nordbring-Hertz, 1985. Microsclerotia germination of *Verticillium dahliae* as affected by rape rhizosphere. *FEMS Microbial Ecology* 31:293-299.
- Ordentlich, A., A. Nachmias, and I. Chet, 1990. Integrated control of *Verticillium dahliae* in potato by *Trichoderma harzianum* and captan. *Crop Protection* 9:363-366.
- Orenstein, J., A. Nachmias, and H. Lips, 1989. Inhibition of tomato root growth in culture by a *Verticillium dahliae* phytotoxin. *Phytoparasitica* 17:269-278.
- Oyarzun, P.J., G. Dijst, F.C. Zoon, and P.W.T. Maas, 1997. Comparison of soil receptivity to *Thielaviopsis basicola*, *Aphanomyces euteiches*, and *Fusarium solani* f.sp. *pisi* causing root rot in pea. *Phytopathology* 87: 534-541.
- Oyarzun, P.J., M. Gerlagh, and J.C. Zadoks, 1998. Factors associated with soil receptivity to some fungal root rot pathogens of peas. *Applied Soil Ecology* 10: 151-169.
- Palloix, A., E. Pochard, T. Phaly, and A.M. Daubeze, 1990. Recurrent selection for resistance to *Verticillium dahliae* in pepper. *Euphytica* 47:79-89.
- Paplomatas, E.J., D.M. Bassett, J.C. Broome, and J.E. DeVay, 1992. Incidence of verticillium wilt and yield losses of cotton cultivar (*Gossypium hirsutum*) based on soil inoculum density of *Verticillium dahliae*. *Phytopathology* 82:1417-1420.
- Paplomatas, E.J., S. Tzavaras, and J.E. Davis, 1997. Use of *Verticillium tricorpus* as a biocontrol of *Rhizoctonia solani* on cotton seedlings. *Book of Abstracts of the 7th International Verticillium Symposium*, Athens, Greece, p. 66.

- Park, C.S., T.C. Paulitz, and R. Baker, 1988. Biocontrol of fusarium wilt of cucumber resulting from interactions between *Pseudomonas putida* and nonpathogenic isolates of *Fusarium oxysporum*. *Phytopathology* 78:190-194.
- Pataky, J.K., and M.K. Beute, 1983. Effects of inoculum burial, temperature, and soil moisture on survival of *Cylindrocladium crotalariae* microsclerotia in North Carolina. *Plant Disease* 67:1379-1382.
- Pegg, G.F., 1974. Verticillium diseases. *Review of Plant Pathology* 53:157-182.
- Pierson, C.F., S.S. Gothoskar, J.C. Walker, and M.A. Stahmann, 1955. Histological studies on the role of pectic enzymes in the development of fusarium wilt symptoms in tomato. *Phytopathology* 45:524-527.
- Pieterse, C.M.J., S.C.M. van Wees, J.A. van Pelt, A. Trijssenaar, Y.A.M. van 't Westende, E.M. Bolink, and L.C. van Loon, 1996. Systemic resistance in *Arabidopsis thaliana* induced by biocontrol bacteria. *Mededelingen van de Faculteit Landbouwwetenschappen Rijksuniversiteit, Gent* 61(2A):209-220.
- Platt, H.W., J. Robb, R. Nazar, and X. Hu, 1995. Verticillium wilt of potatoes: causal agents and pathogen detection. *Phytoparasitica* 23:47.
- Podile, A.R., G.S. Prasad, and H.C. Dube, 1985. *Bacillus subtilis* as antagonist to vascular wilt pathogens. *Current Science* 54:864-865.
- Polak, A., 1989. Melanin as a virulence factor in pathogenic fungi. *Mycoses* 33:215-224.
- Polizzi, G., 1996. Wilt of *Lagunaria patersonii* caused by *Verticillium dahliae*. *Informatore Fitopatologico* 46:46-49.
- Powelson, M.L., and R.C. Rowe, 1993. Biology and management of early dying of potatoes. *Annual Review of Phytopathology* 31:111-126.
- Pullman, G.S., J.E. DeVay, and R.H. Garber, 1981. Soil solarization and thermal death: a logarithmic relationship between time and temperature for four soilborne plant pathogens. *Phytopathology* 71:959-965.
- Pullman, G.S., and J.E. DeVay, 1982. Epidemiology of verticillium wilt of cotton: a relationship between inoculum density and disease progression. *Phytopathology* 72:549-554.
- Raaijmakers, J.M., D.M. Weller, and L.S. Thomashow, 1997. Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments. *Applied and Environmental Microbiology* 63:881-887.
- Raaijmakers, J.M., and D.M. Weller, 1998. Natural plant protection by 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp. in take-all decline soils. *Molecular Plant-Microbe Interactions* 11:144-152.
- Raupach, G.S., and J.W. Kloepper, 1998. Mixture of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. *Phytopathology* 88:1158-1164.
- Resende, M.L.V., J. Flood, and R.M. Cooper, 1994. Host specialization of *Verticillium dahliae*, with emphasis on isolates from cocoa (*Theobroma cacao*). *Plant Pathology* 43:104-111.
- Richardson, M.J., 1990. *An annotated list of seed-borne diseases*, 4th ed. The International Seed Testing Association, Zürich, Switzerland.
- Rijkers, A.J.M., J.A. Hiemstra, and G.J. Bollen, 1992. Formation of microsclerotia of *Verticillium dahliae* in petioles of infected ash trees. *Netherlands Journal of Plant Pathology* 98:261-264.
- Rowe, R.C., J.R. Davis, M.L. Powelson, and D.I. Rouse, 1987. Potato early dying: causal agents and management strategies. *Plant Disease* 71:482-489.
- Rowe, R.C., D.A. Johnson, W.R. Beery, and M.A. Omer, 1997. Vegetative compatibility analysis of strains of *Verticillium dahliae* from potato seed tubers and plants from the western and eastern United States. *Book of Abstracts of the 7th International Verticillium Symposium*, Athens, Greece, p. 23.
- Sackston, W.E., and J.W. Martens, 1959. Dissemination of *Verticillium albo-atrum* on seed of sunflower (*Helianthus annuus*). *Canadian Journal of Botany* 37:759-768.

- Safiyazov, J.S., R.N. Mannanov, and R.K. Sattarova, 1995. The use of bacterial antagonists for control of cotton diseases. *Field Crops Research* 43:51-54.
- Sambrook, J., E.F. Fritsch, and T. Maniatis, 1989. *Molecular cloning, a laboratory manual*, 2nd ed. Book 3. Cold Spring Harbor Laboratory Press, New York.
- Schnathorst, W.C., 1981. Life cycle and epidemiology of *Verticillium*. Pp. 81-111. In: M.E. Mace, A.A. Bell, and C.H. Beckman (Eds.), *Fungal wilt diseases of plants*. Academic Press, New York.
- Schreiber, L.R., and R.J. Green, 1963. Effect of root exudates on germination of conidia and microsclerotia of *Verticillium albo-atrum* inhibited by the soil fungistatic principle. *Phytopathology* 53:260-264.
- Shi, G.Y., G.L. Jian, W.J. Sun, and J. Feng, 1998. Discussion of the research status and direction on cotton verticillium wilt in China. *Acta Phytophylacica Sinica* 25:103-107.
- Sijmons, P.C., F.M.W. Grundler, N. von Mende, P.R. Burrows, and U. Wyss, 1991. *Arabidopsis thaliana* as a new model host for plant-parasitic nematodes. *The Plant Journal* 1:245-254.
- Slattery, R.J., 1981. Inoculum potential of *Verticillium*-infested potato cultivars. *American Potato Journal* 58:135-142.
- Smith, V.L., and R.C. Rowe, 1984. Characteristic and distribution of propagules of *Verticillium dahliae* in Ohio potato field soils and assessment of two assay methods. *Phytopathology* 74:553-556.
- Sosnova, V., and Z. Polak, 1975. Susceptibility of *Arabidopsis thaliana* to infection with some plant viruses. *Biologica Planta* 17:156-158.
- Stapleton, J.J., E.J. Paplomatas, R.J. Wakeman, and J.E. DeVay, 1993. Establishment of apricot and almond trees using soil mulching with transparent (solarization) and black polyethylene film: effects on verticillium wilt and tree health. *Plant Pathology* 42:333-338.
- Stark, C., 1961. Das Auftreten der *Verticillium*-Tracheomykosen in Hamburger Gartenbaukulturen. *Gartenbauwissenschaft* 26:493-528.
- Subbarao, K.V., A. Chassot, T.R. Gordon, J.C. Hubbard, P. Bonello, R. Mullin, D. Okamoto, R.M. Davis, and S.T. Koike, 1995. Genetic relationships and cross pathogenicities of *Verticillium dahliae* isolates from cauliflower and other crops. *Phytopathology* 85:1105-1112.
- Subbarao, K.V., J.C. Hubbard, and S.T. Koike, 1999. Evaluation of broccoli residue incorporation into field soil for verticillium wilt control in cauliflower. *Plant Disease* 83:124-129.
- Susnoschi, M., J. Krikun, and Z. Zuta, 1974. Variety trial of potatoes resistant to verticillium wilt. *American Potato Journal* 52:227-231.
- Tabrett, A.M., J.H. Carder, S. Coomber, and D.J. Barbara, 1995. *Arabidopsis thaliana* as an experimental host of haploid isolates of *Verticillium dahliae*. *Book of Abstracts of the 6th International Verticillium Symposium*, Dead Sea, Israel. *Phytoparasitica* 23:49.
- Taylor, J.B., 1969. Fluctuation in natural soil populations of *Verticillium tricorpus*. *Canadian Journal of Botany* 47:737-740.
- Termorshuizen, A.J., J.R. Davis, G. Gort, D.C. Harris, O.C. Huisman, G. Lazarovits, T. Locke, J.M. Melero Vara, L. Mol, E.J. Paplomatas, H.W. Platt, M. Powelson, D.I. Rouse, R.C. Rowe, and L. Tsrör, 1998. Interlaboratory comparison of methods to quantify microsclerotia of *Verticillium dahliae* in soil. *Applied and Environmental Microbiology* 64:3846-3853.
- Thanassouloupoulos, C.C., 1993. Spread of verticillium wilt by nursery plants in olive groves in the Halkidiki areas (Greece). *Bulletin OEPP* 23:517-520.
- Tjamos, E.C., and D.R. Fravel, 1995. Detrimental effects of sublethal heating and *Talaromyces flavus* on microsclerotia of *Verticillium dahliae*. *Phytopathology* 85:388-392.
- Toyota, K., and M. Kimura, 1993. Colonisation of chlamydospores of *Fusarium oxysporum* f.sp. *raphani* by soil bacteria and their effects on germination. *Soil Biology and Biochemistry* 25:193-197.
- Tsrör, L., O. Erlich, S. Amitai, and M. Hazanovsky, 1998. Verticillium wilt of paprika caused by a highly virulent isolate of *Verticillium dahliae*. *Plant Disease* 82:437-439.
- Tsrör, L., L. Livescu, and A. Nachmias, 1990. Effect of light duration and growth season on verticillium wilt in potato. *Phytoparasitica* 18:331-339.

- Tsuji, J., and S.C. Somerville, 1992. First report of natural infection of *Arabidopsis thaliana* by *Xanthomonas campestris* pv. *campestris*. *Plant Disease* 76:539.
- Van der Koppel, M.M., and A. Schots, 1995. Monoclonal antibody-based double-antibody sandwich-ELISA for detection of *Verticillium* spp. in ornamentals. *Phytopathology* 85:608-612.
- Van der Spek, J., 1972. Internal carriage of *Verticillium dahliae* by seeds and its consequences. *Mededelingen Faculteit Landbouwwetenschappen Rijksuniversiteit Gent*, 37:567-573.
- Van Loon, L.C., 1997. Induced resistance in plants and the role of pathogenesis-related proteins. *European Journal of Plant Pathology* 103:753-765.
- Whalen, M.C., R.W. Innes, A.F. Bent, and B.J. Staskawicz, 1991. Identification of *Pseudomonas syringae* pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidopsis* and soybean. *The Plant Cell* 3:49-59.
- Wheeler, T.A., and R. Rowe, 1995. Influence of soil characteristics and assay techniques on quantification of *Verticillium dahliae* in Ohio soils. *Plant Disease* 79: 29-34.
- Wilhelm, S., 1951. Is *Verticillium albo-atrum* a soil invader or a soil inhabitant? *Phytopathology* 41: 944-945.
- Wilhelm, S., 1955. Longevity of the verticillium wilt fungus in the laboratory and field. *Phytopathology* 45:180-181.
- Williams, R.H. and J.M. Whipps, 1995. Growth and transmission of *Coniothyrium minitans* in soil. *IOBC/WPRS Bulletin* 18:39-43.
- Winston, P.W., and D.H. Bates, 1960. Saturated solutions for the control of humidity in biological research. *Ecology* 41:232-237.
- Xiao, C.L., K.V. Subbarao, K.F. Schulbach, and S.T. Koike, 1998. Effects of crop rotation and irrigation on *Verticillium dahliae* microsclerotia in soil and wilt in cauliflower. *Phytopathology* 88:1046-1055.
- Zeise, K., 1997. The potential of *Talaromyces flavus* (Klöcker) Stolk & Samson in controlling *Verticillium dahliae*. *Book of Abstracts of the 7th International Verticillium Symposium*, Athens, Greece, p. 61.
- Zilberstein, Y., I. Chet, and Y. Henis, 1983. Influence of microsclerotia source of *Verticillium dahliae* on inoculum quality. *Transactions of the British mycological Society* 81:613-617.

SUMMARY

The soilborne pathogen *Verticillium dahliae* Kleb. causes wilt in a wide range of hosts. It affects many crops, the most economically significant being cotton, potato, and olive. In addition many other crops can be affected seriously, including vegetables, fruits, fruit trees, fibre, oil-seed crops, ornamentals and shade trees. Generally monocotyledonous plants are considered non-hosts with the notable exception of barley. The pathogen occurs world-wide in the temperate and subtropical zones of all continents, but only sporadically in the tropics. It forms highly persistent survival structures, the microsclerotia, which allow extended survival in the absence of hosts. Given the wide host range of *V. dahliae*, crop rotation provides only limited protection against the disease, an alternation with monocotyledonous crops such as cereals yielding the best results. Control of verticillium wilt has largely been based on chemical soil disinfestation but, because of nonselective modes of action and environmental impact, such use is increasingly restricted by national governments. However, no economic alternatives are currently available except for soil solarization in the Mediterranean areas. Microsclerotia are generally regarded as the target structures to be controlled through strategies of biological or cultural control, but their dynamics and ecology within the life cycle of *V. dahliae* have only rarely been studied in detail. The factors affecting formation, survival, and death of microsclerotia of *V. dahliae* are the topics of this thesis.

In a first part fundamental ecological studies were directed on the formation and survival of microsclerotia. Detailed quantitative knowledge about factors influencing these processes may lead to new prospects to control. In a second part an improvement of the control of verticillium wilt by use of biocontrol agents was attempted. We hypothesized that the above-ground application of the biocontrol agent *Talaromyces flavus* onto microsclerotia-containing debris would reduce the inoculum density of *V. dahliae*. In addition, the effects of adding a diacetylphloroglucinol-producing strain of *Pseudomonas fluorescens* on verticillium wilt were studied. This strain had been found to be effective in suppressing take-all in wheat, caused by *Gaeumannomyces graminis*.

Arabidopsis thaliana was found to be a suitable bioassay plant to detect low soil-borne inoculum levels of *V. dahliae* (Chapter 2). Although crucifers are predominantly infected in the field by *V. longisporum*, *A. thaliana* was readily infected under the test conditions by four isolates of *V. dahliae*. Disease symptoms consisted of early senescence, but no typical verticillium wilt symptoms were observed. Increasing amounts of infection of both root and shoot were noted at densities of 1, 3, 10, 30, and 100 microsclerotia g⁻¹ soil. A log-linear relation

between inoculum density and the area-under-the-disease progress curve was established. Even at the lowest inoculum density tested of 1 microsclerotium g^{-1} soil, 5% of the root length became infected with *V. dahliae*, and 30% of the stem. The most sensitive measure was the amount of new microsclerotia formed in the shoot. The higher the inoculum density the greater the density of microsclerotia in the shoot g^{-1} shoot dry weight, indicating that multiple root infections lead to a more intense colonization of *V. dahliae*. Disease progress was fastest at 20°C, and slower at 10, 15, and 25°C. This bioassay appears particularly suited for assessing whether a field soil is infested with *V. dahliae*.

The formation of microsclerotia of six isolates of *V. dahliae* was studied at different temperatures both *in vitro* and on *A. thaliana* (Chapter 3). Seedlings of *A. thaliana* were root-dipped in a conidial suspension, planted, and either placed at 5, 10, 15, or 25°C, or left at 20°C until the onset of senescence, after which part of the plants was transferred to 5, 10, 15, or 25°C. Generally, the optimal temperature for production of microsclerotia was 20°C. Two isolates produced about ten times more microsclerotia than each of the other four isolates. In a multiple regression analysis for these isolates high $R^2_{\text{adj.}}$ -values of 0.77 and 0.66 were obtained in multiple regressions, with temperature and its square as highly significant ($P < 0.001$) independent variables. $R^2_{\text{adj.}}$ -values for the other isolates varied between only 0.28 and 0.39. The act of moving plants to different temperatures at the onset of senescence led to microsclerotial densities that were intermediate to plants that had grown at constant temperatures. This suggests that vascular colonization rate and rate of formation of microsclerotia are equally affected by temperature. The senescence rate of plants appeared unimportant except for plants grown at 25°C, which showed highest amounts of microsclerotia per unit of plant weight in the most rapidly senescing plants. It is concluded that temperature and isolate are primary factors involved in the formation of microsclerotia in host tissue.

In Chapter 4, the influence of various conditions on the survival of microsclerotia of *V. dahliae* was studied using field-collected potato stems containing dense masses of naturally-formed microsclerotia. The survival of microsclerotia for up to two years in potato stems not incorporated in soil was studied under various conditions of temperature and relative humidity. The effects of temperature, pF, including weekly variations in temperature and/or pF, and various modes of incorporating potato stem tissue on the survival of microsclerotia for up to one year in a sandy unsterilized soil was also studied for microsclerotia from different sources. No significant isolate effect was found. The recovery of microsclerotia one day after the start of experiments was remarkably low, varying between 5.5 and 31% of the number added to the soil. Recovery continued at this level or even lower in all experiments for 1 month incubation and, with several treatments, also after 3 and 6 months. After 3 to 12 months, this decline in recovery

was frequently followed by an up to 5 times increase in recovery. These recoveries did not exceed the number of microsclerotia initially incorporated into the soil. Changes in recovery may be due to variation in the level of soil fungistasis which is affected by the rate of nutrient leakage from microsclerotia.

Chapter 5 deals with the effects of application of the antagonist *Talaromyces flavus* onto potato stems infected with *V. dahliae* on the survival and inoculum potential of pathogen. The antagonist *T. flavus* was applied onto potato stems infected with *Verticillium dahliae* and its effects on survival and inoculum potential of microsclerotia of the plant pathogen were evaluated. Three weeks after application of 3 g alginate prill formulation of *T. flavus* on 150 1-cm-long pieces of potato stems, the antagonist was growing prominently over the potato stems at 25°C but not at 15°C. Germinability of microsclerotia was reduced only in the three experiments carried out at 25°C and not in two experiments at 15°C. After 21 days of above-ground incubation, 15 potato stems were incorporated into a sand/potting soil mixture. Populations of *T. flavus* and *V. dahliae* were determined 4 and 10 months after incubation. The population level of *T. flavus* was significantly ($P < 0.05$) higher in the treatments where after its application the potato stems were left 21 days above-ground compared to direct incorporation of the stems + *T. flavus* into the soil. This was attributed to the relatively low levels of competition occurring above-ground. The population level of *T. flavus* persisted at at least 240 colony-forming units g^{-1} soil, and showed a 10 to 12-times increase after 10 months compared to 4 months after incubation at 25°C. Population levels of *V. dahliae* were significantly decreased by *T. flavus* at both incubation temperatures. Leaving *T. flavus*-treated potato stems above-ground for 21 days did not affect the survival of microsclerotia compared to direct incorporation of the treated potato stems into the soil. A significant inverse relation between population size of *T. flavus* and that of *V. dahliae* was found only for incubation at 25°C. To determine the inoculum potential of *V. dahliae*, 4-week-old seedlings of *Arabidopsis thaliana* were planted either directly after incorporation of the potato stems in soil at 20°C; or soils were left another 21 days at 15 or 25°C before the bioassay with *A. thaliana* was carried out at 20°C. Temperature did not affect the development of senescence of *A. thaliana*, which was retarded significantly for all *T. flavus* treatments. This could be due to relatively high levels of *T. flavus* occurring in all experiments. It is concluded that application of *T. flavus* on fresh organic debris containing microsclerotia reduces inoculum densities of *V. dahliae*. The effect of population density of *T. flavus* on the rate of development of wilt symptoms caused by *V. dahliae* warrants more research.

In addition to *T. flavus*, effects of a 2,4-diacetylphloroglucinol-producing strain of *Pseudomonas fluorescens* were examined in Chapter 6. *P. fluorescens* strain P60 inhibited mycelial growth of 20 isolates of *V. dahliae* *in vitro* by at least 50% and also the formation of

microsclerotia was significantly reduced by P60 for the majority of the isolates tested. In bioassays, *V. dahliae* significantly accelerated senescence of *A. thaliana* and eggplant. However, when plants were treated with P60, senescence progressed at the same rate as in the treatment without *V. dahliae*. Although treatment of plants with P60 did not lead to major reductions in the percentage of infected shoots of *A. thaliana*, the amount of microsclerotia produced by *V. dahliae* on senesced shoots was significantly reduced (up to almost 8-fold) in all four experiments. *P. fluorescens* could not be isolated from the interior of the host tissue, but it was present in the rhizosphere of both *A. thaliana* and eggplant up to 12 weeks after inoculation at densities ranging from 1.4×10^3 - 8.0×10^5 cfu g⁻¹ roots. Combination of P60 with *T. flavus* further improved control of *V. dahliae*. Compared to the control treatment, the amount of microsclerotia formed was reduced up to almost 40-fold by combined application of P60 and *T. flavus*, and control obtained by the combination was significantly better than the reductions obtained by applications of each agent separately.

Three main issues are identified in the general discussion (Chapter 7): (1) the usefulness of applying bioassays to quantify the inoculum potential of *V. dahliae*, (2) factors affecting the number of microsclerotia in soil, and (3) biocontrol of *V. dahliae*. Bioassays essentially provide different information from plating techniques that only enumerate the microsclerotia. The bioassay presented appears to be highly sensitive and seems well-suited to detect low soil infestations that are difficult to detect by other methods. Moreover, it is very suitable for use in comparative, experimental studies. Factors affecting the number of microsclerotia in soil can be divided into those affecting the formation of new microsclerotia, and those affecting survival and recovery of the fungus. In determining the density of microsclerotia in soil, recovery may play an important, confounding, role. Suggestions are made for further studies in this field, with special emphasis on the question how low recovery rates may affect the interpretation of processes in soil. There are clear opportunities for developing methods for controlling verticillium wilt using antagonists. It is recommended that different strains of both *P. fluorescens* and *T. flavus* against verticillium wilt are screened. It is supposed that the reduced formation of new microsclerotia in shoot tissue induced by both antagonists is due to a reduced number of root infections of *V. dahliae*, or to induced resistance.

SAMENVATTING

Verticillium dahliae is een bodemschimmel die verwelkingsziekte veroorzaakt bij tal van gewassen. De belangrijkste waardplanten zijn katoen, aardappel en olijf, maar een groot aantal andere, economisch belangrijke gewassen kunnen worden aangetast, waaronder aardbei, roos, esdoorn, es en chrysant. Monocotyle gewassen, zoals granen, zijn geen waardplanten, hoewel gerst hierop een uitzonderling is. *V. dahliae* komt wereldwijd voor in gebieden met een gematigd of subtropisch klimaat. De schimmel vormt kleine (diameter ca. 60 µm), meercellige, zwarte overlevingsstructuren die microsclerotien genoemd worden. Gezien de grote waardplantenreeks kan vruchtwisseling met granen slechts in geringe mate bijdragen tot beheersing van de verwelkingsziekte. Bestrijding van verticillium-verwelkingsziekte is tot nu toe gebaseerd geweest op chemische grondontsmetting, maar de toelating voor deze bestrijdingsmethode wordt steeds meer ingeperkt, omdat grondontsmetting te weinig selectief is, en effecten kan hebben op de kwaliteit van het grond- en oppervlaktewater, alsmede de ozonlaag. Op dit moment zijn er weinig andere maatregelen ter beheersing van de verwelkingsziekte beschikbaar. In landen met een mediterraan klimaat wordt solarisatie uitgevoerd. Hierbij wordt vochtige grond afgedekt met plastic, waarna in betrekkelijk korte tijd de temperatuurstijging doding van *V. dahliae* teweegbrengt. Hoewel reductie van het aantal microsclerotien in de bestrijding van verwelkingsziekte nagestreefd wordt, is de dynamiek en ecologie van deze structuren onvoldoende bekend. Daarom zijn de factoren die de vorming, overleving, en sterfte van microsclerotien van *V. dahliae* beïnvloeden onderwerp van dit proefschrift.

Het eerste doel van dit proefschrift was fundamenteel-ecologische kennis over vorming en overleving van microsclerotien te verwerven. Kennis van kwantitatieve effecten van factoren die deze processen beïnvloeden zou kunnen leiden tot nieuwe benaderingen voor de beheersing van verticillium-verwelkingsziekte. Het tweede doel was de biologische bestrijding van de ziekte met de antagonisten *Talaromyces flavus* en *Pseudomonas fluorescens*. We verwachtten dat bovengrondse toepassing van de bodemschimmel *T. flavus* op gewasresten die microsclerotien bevatten zou leiden tot een verbeterde bestrijding van de ziekte. Verder werden de effecten van phloroglucinol-producerende stammen van *P. fluorescens* op verticillium-verwelkingsziekte onderzocht.

In hoofdstuk 2 werd de mogelijkheid van gebruik van *Arabidopsis thaliana* als toetsplant ter bepaling van de dichtheid van microsclerotien van *V. dahliae* in de grond onderzocht. Hoewel in het veld cruciferen, waartoe *A. thaliana* behoort, hoofdzakelijk door *V. longisporum* worden geïnfecteerd, bleek de plant ook te kunnen worden geïnfecteerd door *V. dahliae*. Het

ziektesymptoom bestond uit vervroegde verwelking, maar voor verticillium-verwelkingsziekte typische symptomen werden niet waargenomen. Het effect van verschillende dichtheden van microsclerotieën van *V. dahliae* op de infectie van wortel en stengel van *A. thaliana* werd onderzocht. De hoeveelheid infectie van wortel en stengel nam toe bij toenemende dichtheid van microsclerotieën en een log-lineaire relatie werd vastgesteld tussen inoculumdichtheid en oppervlak onder de ziektevoortschrijdingscurve. Zelfs bij de laagste inoculumdichtheid van 1 microsclerotium per gram grond werd 5% wortelinfectie en 30% stengelinfectie gevonden. De meest gevoelige parameter bleek de hoeveelheid nieuwgevormde microsclerotieën per gram spruitweefsel te zijn. De ziekte ontwikkelde zich het snelst bij 20°C, en minder bij 10, 15 en 25°C. De biotoets lijkt vooral nuttig te zijn bij de vaststelling van lage inoculumdichtheden van *V. dahliae* in de grond.

De vorming van microsclerotieën van zes isolaten van *V. dahliae* in *A. thaliana* werd onderzocht bij verschillende temperaturen. In reïncultuur bleek de myceliumgroei het snelst te zijn bij 25°C, maar de productie van microsclerotieën het grootst bij 20°C (twee isolaten) of 15-20°C (één isolaat). Kiemplanten werden gedoopt in een conidiënsuspensie van *V. dahliae* en bij 5, 10, 15, 20, of 25°C geplaatst. Een deel van de planten werd eerst bij 20°C geplaatst, en pas op het moment dat de productie van microsclerotieën bijna begon geplaatst bij 5, 10, 15, of 25°C. De hoeveelheid nieuwgevormde microsclerotieën bleek afhankelijk van isolaat en temperatuur. In het algemeen was de optimale temperatuur voor productie van microsclerotieën 20°C. Twee isolaten produceerden ongeveer tien maal meer microsclerotieën per eenheid plantgewicht dan de andere isolaten. Voor deze twee isolaten kon de vorming van microsclerotieën per eenheid plantgewicht voorspeld worden met de temperatuur en het kwadraat van de temperatuur. De vorming van microsclerotieën bij de andere isolaten kon echter niet worden voorspeld aan de hand van de temperatuur of andere parameters zoals plantgewicht en effect van *V. dahliae*. Het verplaatsen van planten op het moment dat de productie van microsclerotieën bijna startte van 20°C naar 5, 10, 15, of 25°C leidde tot hoeveelheden microsclerotieën per eenheid plantgewicht die lagen tussen die van planten die continu bij 20°C opgegroeid waren en die welke continu bij de andere temperaturen opgegroeid waren. Dit suggereert dat temperatuur zowel een groot effect heeft op de ontwikkeling van *V. dahliae* in het vaatstelsel als op de vorming van microsclerotieën. De snelheid waarmee de plantjes afrijpten bleek ongecorreleerd te zijn met de productie van microsclerotieën, behalve bij de planten die waren opgegroeid bij 25°C. Daar werd een negatief verband gevonden, hetgeen aangeeft dat de meeste microsclerotieën worden gevormd bij planten die het snelst afrijpen.

In hoofdstuk 4 werd het effect van temperatuur en bodemvochtgehalte op de overleving van microsclerotieën onderzocht. Hiertoe werden verschillende collecties van uit het veld

verzamelde, op aardappelstengels gevormde, microsclerotiën in grond gebracht en gedurende één tot twee jaar geïncubeerd onder verschillende omstandigheden. Hoewel een groot aantal extreme behandelingen werden uitgevoerd, waaronder bijvoorbeeld wekelijkse veranderingen in watergehalte en/of temperatuur, was er grote overleving na één tot twee jaar in praktisch alle behandelingen. Er werd geen effect gevonden van de verschillende collecties van microsclerotiën. Eén dag nadat de microsclerotiën in de grond waren gebracht, konden slechts 5.5-31% van het totale aantal teruggevonden worden. Dit percentage bleek nog lager voor alle behandelingen één maand na begin van de experimenten, en voor verscheidene behandelingen nog lager drie en zes maanden na begin van de experimenten. Na deze initiële daling nam het aantal microsclerotiën echter toe, veelal tot niveaus hoger dan de hoeveelheid die waargenomen werd één dag na start van de experimenten, maar lager dan de oorspronkelijk toegediende hoeveelheid. In de discussie werd geopperd dat deze resultaten niet zozeer te verklaren zijn uit sterfte en groei van *V. dahliae* in de grond, maar eerder op basis van variaties in de sterkte van de bodemfungistase, die zou kunnen worden beïnvloed door de mate van exsudatie van voedingsstoffen uit microsclerotiën, waarbij jonge microsclerotiën meer zouden exsuderen, en dus gevoeliger zouden zijn voor bodemfungistase, dan oudere.

In hoofdstuk 5 werd getracht een consistentere biologische bestrijding te krijgen met de antagonist *Talaromyces flavus* door met microsclerotiën bezette stukjes aardappelstengels bovengronds te behandelen met een formulering van de antagonist. Na drie weken incubatie bleek de kieming van microsclerotiën significant te zijn gereduceerd bij 25°C, maar niet bij 15°C. Vervolgens werden de wel of niet met *T. flavus* behandelde aardappelstengels in de grond gebracht. Een ander deel werd met *T. flavus* behandeld en onmiddellijk in de grond gebracht. De populatiedichtheid van *T. flavus* bleek vier en tien maanden na incubatie significant hoger te zijn in de behandeling waarbij de aardappelstengels eerst drie weken bovengronds was geïncubeerd. De populatiedichtheid van *T. flavus* nam in alle behandelingen sterk toe bij 25°C, maar niet bij 15°C. Populatiedichtheden van *V. dahliae* waren significant lager in aanwezigheid van *T. flavus*, maar er was geen effect van het gedurende drie weken bovengronds laten incuberen van met de antagonist behandelde aardappelstengels. Een biotoets werd uitgevoerd met *A. thaliana* op de twee behandelingen (directe toevoeging aan de grond en incubatie gedurende drie weken van de aardappelstengels gevolgd door toevoeging aan de grond), waarbij kiemplanten direct in de grond werden geplant, of pas drie weken later. Er werd een duidelijk, significant effect van *T. flavus* op de ontwikkeling van verticillium-verwelkingsziekte waargenomen bij zowel 15 als 25°C, maar andere effecten (temperatuur, incubatie bovengronds) werden niet waargenomen. Dit zou kunnen wijzen op geïnduceerde resistentie als mechanisme van ziekte-onderdrukking door *T. flavus*. Aangezien bovengrondse toepassing van *T. flavus* leidt tot een sterkere toename van de

biomassa van de antagonist werd geconcludeerd dat een dergelijke werkwijze zou kunnen leiden tot een meer consistente bestrijding van de verticillium-verwelkingsziekte.

In hoofdstuk 6 werd het effect van de bacterie *Pseudomonas fluorescens* op de verticillium-verwelkingsziekte onderzocht. In het bijzonder werd isolaat P60 onderzocht, die het antibioticum 2,4-diacetylphloroglucinol produceert. Dit isolaat werd verkregen uit een grond waarop continu tarwe werd geteeld, en waar spontane achteruitgang van de tarwehalmdoder, *Gaeumannomyces graminis*, is waargenomen. Dit isolaat, alsmede twee andere, veroorzaakten op agarmedium een reductie in groei van minimaal 50%, alsmede significante reductie in de vorming van microsclerotiën voor de meeste van de 20 onderzochte isolaten van *V. dahliae*. Het effect van *P. fluorescens* P60 werd onderzocht in biotoetsen met *A. thaliana* en aubergine op potgrond en een uit het veld verzamelde lemige zandgrond. Behandeling van kiemplanten door dopen in een suspensie van *P. fluorescens* vertraagde de veroudering van de planten op grond met 50 microsclerotiën per gram grond, zodat die gelijk was aan die van de controle zonder microsclerotiën. Hoewel het infectiepercentage in alle behandelingen hoog was (100% in de controles en 75-100% in de behandelingen met *P. fluorescens*), bleek de vorming van nieuwe microsclerotiën in de spruit van afgerijpte *A. thaliana* $4.1-7.5 \times$ te zijn gereduceerd. De bacterie bleek zich te handhaven in de rhizosfeer, maar kon niet worden geïsoleerd uit plantenweefsel. Combinatie van *P. fluorescens* met *T. flavus* leidde tot een geringe vermindering van de ziekte, en tot een verdere vermindering van de hoeveelheid nieuw-gevormde microsclerotiën. Mogelijk beschermen de twee antagonisten verschillende delen van de wortel.

In de algemene discussie (hoofdstuk 7) werd de biotoets, de dichtheid van microsclerotiën in de grond, en de biologische bestrijding van de verticillium-verwelkingsziekte besproken. Biotoetsen leveren informatie die anders is dan de informatie die voortkomt uit bepalingen van de dichtheid van de microsclerotiën in de grond door uitplaattechnieken. Met biotoetsen worden problemen met uitplaattechnieken omzeild, maar de resultaten van biotoetsen zijn in het algemeen variabel en de inoculumpotentiaal, de maat die bij biotoetsen wordt vastgesteld, is moeilijk te interpreteren. De hier voorgestelde biotoets is echter zeer gevoelig en kan goed dienst doen bij de vaststelling van geringe besmettingen van de grond met *V. dahliae*. Factoren die het aantal microsclerotiën in de grond beïnvloeden betreffen de nieuwe vorming en de overleving. Bij de bepaling van het aantal microsclerotiën in de grond speelt de mate van terugwinning uit de grond een belangrijke rol. Enkele suggesties voor verder onderzoek werden gedaan, waarbij de nadruk werd gelegd op de vraag of de lage terugwinningspercentages relevant zijn voor biologische processen in de grond. Dit onderzoek opent veelbelovende vooruitzichten voor biologische bestrijding van *V. dahliae*. Aanbevolen wordt een verdere selectie te maken uit een groot aantal isolaten van *P. fluorescens* en *T. flavus*.

RINGKASAN

Patogen tular-tanah *Verticillium dahliae* Kleb. menyebabkan penyakit layu dengan kisaran tanaman inang yang luas. *V. dahliae* mempengaruhi pertumbuhan banyak tanaman, dan yang nyata sangat penting secara ekonomi adalah tanaman kapas, kentang dan zaitun. Selain itu, pertumbuhan tanaman-tanaman lainnya termasuk sayur, buah, serat dan penghasil minyak, serta hias dan pepohonan dapat secara serius dipengaruhi oleh jamur ini. Pada umumnya, tanaman berkeping tunggal tidak dianggap sebagai tanaman inangnya, tetapi suatu perkecualian telah dilaporkan untuk tanaman "barley". Patogen tersebar luas di daerah beriklim sedang dan subtropika di semua benua, tetapi hanya terserak di daerah tropika. Patogen membentuk struktur untuk bertahan hidup yang tinggi persistensinya, yaitu mikrosklerotium, yang mampu bertahan hidup di dalam tanah dalam jangka waktu lama tanpa adanya tanaman inang.

Berdasarkan kisaran inang jamur *V. dahliae* yang luas, maka pergiliran tanaman hanya mampu melindungi tanaman secara terbatas terhadap penyakit yang ditimbulkannya, dengan satu pilihan terbaik pada tanaman berkeping tunggal, seperti sereal. Pengendalian penyakit layu vertisilium secara luas telah didasarkan pada disinfestasi tanah secara kimia. Akan tetapi, dikarenakan cara kerjanya yang tidak khusus pada organisme tertentu dan pengaruh negatifnya terhadap lingkungan, maka pelarangan penggunaannya secara nasional oleh pemerintah setempat makin meningkat. Penggantian cara pengendalian yang ekonomis di masa sekarang belum tersedia selain dengan pematihan tanah di daerah Mediterania. Mikrosklerotium umumnya dianggap sebagai struktur sasaran yang harus dikendalikan melalui beberapa strategi, seperti pengendalian secara hayati dan cara bercocok tanam, tetapi dinamika dan ekologi tentang mikrosklerotium di dalam daur hidup *V. dahliae* jarang dikaji secara rinci. Faktor-faktor yang mempengaruhi pembentukan, cara bertahan hidup, dan kematian mikrosklerotium *V. dahliae* adalah menjadi pokok kajian di dalam tesis ini.

Pada bagian pertama, kajian secara ekologi tentang pengetahuan dasar ditujukan terhadap pembentukan dan cara bertahan hidup mikrosklerotium. Pengetahuan kuantitatif tentang faktor-faktor yang mempengaruhi proses ini secara rinci kemungkinan ditujukan untuk menemukan prospek baru dalam pengendalian suatu patogen. Pada bagian kedua, peningkatan pengendalian layu vertisilium dengan menggunakan agensia pengendali hayati dilakukan. Kami menghipotesiskan bahwa penerapan agensia pengendali hayati *Talaromyces flavus* di permukaan tanah, terhadap sisa-sisa tanaman yang mengandung mikrosklerotium, akan mengurangi kepadatan inokulum *V. dahliae*. Selain itu, juga dikaji pengaruh penambahan strain bakteri *Pseudomonas fluorescens* penghasil antibiotika 2,4-diacetylphloroglucinol terhadap penyakit layu vertisilium. Strain ini telah ditemukan secara efektif menekan penyakit "take-all" pada gandum, yang disebabkan oleh jamur *Gaeumannomyces graminis*.

Arabidopsis thaliana diketahui sesuai sebagai tanaman uji hayati untuk mendeteksi tingkat inokulum yang rendah dari tular-tanah *Verticillium dahliae* (Bab 2). Walaupun tumbuhan golongan krusifer ini di lapang telah diketahui nyata diinfeksi oleh *V. longisporum*, tetapi pada kondisi uji, *A. thaliana* dapat diinfeksi oleh empat jenis isolat *V. dahliae*. Gejala penyakit yang ditimbulkannya adalah termasuk penuaan awal, sedangkan gejala layu vertisilium yang khas tidak nampak. Banyaknya infeksi pada akar dan tanaman bagian atas yang meningkat terjadi pada kepadatan 1, 3, 10, 30, dan 100 mikrosklerotium per gram tanah. Hubungan log-linier antara kepadatan inokulum dan daerah di bawah lengkung kemajuan penyakit adalah nyata. Bahkan pada kepadatan inokulum terendah yang diuji, yaitu 1 mikrosklerotium per gram tanah, 5% dari panjang akar dan 30% dari batang tanaman menjadi terinfeksi oleh *V. dahliae*. Pengukuran yang paling peka adalah dengan mengetahui banyaknya mikrosklerotium baru yang dibentuk pada tanaman bagian atas. Makin tinggi kepadatan inokulum, maka makin besar kepadatan mikrosklerotium pada tanaman bagian atas per gram berat keringnya, yang menunjukkan bahwa telah terjadi penggandaan infeksi akar yang menuju pada makin seringnya pengkolonian oleh jamur *V. dahliae*. Laju kecepatan penyakit terjadi sangat cepat pada suhu 20°C, dan lebih rendah pada suhu 10, 15, dan 25°C. Uji hayati ini nampaknya secara khusus sesuai untuk menilai apakah suatu tanah lapang terinfeksi oleh *V. dahliae*.

Pembentukan mikrosklerotium dari enam isolat *V. dahliae* dikaji pada suhu yang berbeda secara uji *in vitro* dan pada *A. thaliana* (Bab 3). Akar semai *A. thaliana* direndam di dalam suatu larutan konidium, kemudian ditanam, dan ditempatkan baik pada suhu 5, 10, 15, dan 25°C atau pada suhu 20°C sampai roset mengalami penuaan, dan sesudahnya dipindahkan ke suhu 5, 10, 15, atau 25°C. Pada umumnya, suhu optimum untuk menghasilkan mikrosklerotium adalah 20°C. Dua isolat jamur ini masing-masing mampu menghasilkan mikrosklerotium kira-kira 10 kali lebih banyak bila dibandingkan dengan keempat isolat lainnya. Pada suatu analisis regresi ganda untuk isolate-isolate ini diperoleh nilai R^2_{adj} yang tinggi, yaitu 0,77 dan 0,66, dengan suhu dan nilai kuadratnya sebagai peubah tetap yang sangat nyata ($P < 0,001$). Nilai R^2_{adj} untuk isolate-isolate lainnya hanya beragam antara 0,28 dan 0,39. Kegiatan pemindahan tanaman ke suhu yang berbeda pada saat roset daun mengalami penuaan menyebabkan kepadatan mikrosklerotium yang nampak di tengah jika dibandingkan dengan tanaman yang ditumbuhkan pada suhu tetap. Hal tersebut memberikan dugaan bahwa laju kecepatan pengkolonian jaringan vaskuler dan pembentukan mikrosklerotium adalah sama-sama dipengaruhi oleh suhu. Laju kecepatan penuaan tanaman nampak tidak begitu penting kecuali untuk tanaman yang tumbuh pada suhu 25°C, yang memperlihatkan jumlah mikrosklerotium tertinggi per unit berat tanaman pada tanaman yang mengalami penuaan paling cepat. Hal ini disimpulkan bahwa suhu dan isolat merupakan faktor utama yang berperan dalam pembentukan mikrosklerotium di dalam jaringan inang.

Pada Bab 4, kajian tentang pengaruh berbagai kondisi terhadap daya tahan hidup mikrosklerotium jamur *V. dahliae* dilakukan dengan menggunakan batang tanaman kentang yang dikumpulkan dari lapang, yang mengandung kepadatan mikrosklerotium tinggi dan terbentuk secara alami. Daya tahan hidup mikrosklerotium sampai selama dua tahun di dalam batang tanaman kentang yang tidak ditanam ke dalam tanah dikaji pada berbagai kondisi suhu dan kelembapan relatif. Juga dikaji pengaruh suhu, pF, termasuk keragaman mingguan pada suhu dan/atau pF, dan berbagai cara penanaman jaringan batang tanaman kentang terhadap daya tahan hidup mikrosklerotium dari berbagai sumber sampai selama satu tahun di dalam tanah berpasir yang tidak disterilkan. Hasilnya, tidak dijumpai pengaruh isolat secara nyata. Pemulihan mikrosklerotium dalam waktu sehari setelah dimulainya penelitian adalah sangat rendah, yang beragam antara 5,5 dan 3,1% dari jumlah mikrosklerotium yang ditambahkan ke dalam tanah. Pemulihan berlanjut pada tingkat ini atau bahkan lebih rendah di semua penelitian setelah satu bulan inkubasi, dengan beberapa perlakuan, juga setelah 3 dan 6 bulan. Setelah 3 sampai 12 bulan, penurunan pemulihan ini sering diikuti oleh peningkatan pemulihan sampai 5 kali lebih tinggi. Akan tetapi, pemulihan ini belum pernah meluas pada jumlah mikrosklerotium mula-mula yang ditanam ke dalam tanah, yang menunjukkan bahwa sepertinya tidak ada pembentukan mikrosklerotium baru. Perubahan yang terjadi ini kemungkinan disebabkan oleh keragaman di dalam tingkat fungistasis di dalam tanah yang dipengaruhi oleh laju pelepasan nutrisi dari mikrosklerotium.

Bab 5 berkaitan dengan pengaruh penerapan antagonis *Talaromyces flavus* pada batang tanaman kentang yang terinfeksi oleh *V. dahliae* terhadap daya tahan dan potensi inokulum patogen. Antagonis *T. flavus* diterapkan pada permukaan batang tanaman kentang yang terinfeksi oleh *V. dahliae*, dan pengaruhnya terhadap daya tahan hidup dan potensi inokulum mikrosklerotium patogen tanaman diuji. Tiga minggu setelah penerapan 3 g, *T. flavus* yang diformulasikan dalam butiran "alginate" terhadap 150 keping batang kentang yang dipotong sepanjang 1 cm mampu tumbuh secara tetap di permukaan batang kentang pada suhu 25°C, tetapi tidak pada suhu 15°C. Pengurangan perkecambahan mikrosklerotium hanya terjadi di tiga penelitian pada suhu 25°C dan tidak pada dua penelitian di suhu 15°C. Setelah penginkubasian selama 21 hari di permukaan tanah, 15 keping batang tanaman kentang ditanam ke dalam pot berukuran 400 cm³ yang berisi campuran antara pasir dan tanah pot. Populasi *T. flavus* dan *V. dahliae* diteliti selama 4 dan 10 bulan setelah inkubasi. Tingkat populasi *T. flavus* adalah nyata lebih tinggi ($P < 0,05$) pada perlakuan batang tanaman kentang yang ditanam selama 21 hari di permukaan tanah jika dibandingkan yang langsung ditanam ke dalam tanah. Hal ini memperlihatkan rendahnya tingkat persaingan di permukaan tanah. Tingkat populasi *T. flavus* bertahan pada teratur pada paling sedikit 240 unit pembentuk koloni (upk) per gram tanah, dan memperlihatkan peningkatan sebesar 10 sampai 12 kali setelah 10 bulan bila dibandingkan

dengan setelah diinkubasikan selama 4 bulan pada suhu 25°C. Tingkat populasi *V. dahliae* menurun secara nyata karena *T. flavus* di kedua suhu penginkubasian. Meninggalkan keping batang tanaman kentang yang telah diperlakukan dengan *T. flavus* dan dibiarkan di permukaan tanah selama 21 hari tidak mempengaruhi daya tahan hidup mikrosklerotium, jika dibandingkan dengan pembenaman langsung batang tersebut ke dalam tanah. Hubungan terbalik yang nyata antara ukuran populasi *T. flavus* dan *V. dahliae* hanya dijumpai untuk penginkubasian pada suhu 25°C. Semai *Arabidopsis thaliana* berumur empat minggu ditanam untuk menentukan potensi inokulum *V. dahliae*, baik secara langsung setelah pembenaman keping batang tanaman kentang ke dalam tanah pada suhu 20°C atau ke tanah yang dibiarkan dahulu selama 21 hari lagi pada suhu 15 maupun 25°C sebelum uji hayati dengan *A. thaliana* dilakukan pada suhu 20°C. Hal ini dilakukan untuk meneliti potensi inokulum jamur *V. dahliae*. Perkembangan penuaan *A. thaliana* tidak dipengaruhi oleh suhu, tetapi dihambat secara nyata oleh semua perlakuan dengan *T. flavus*. Hal ini dapat disebabkan oleh tingkat *T. flavus* yang relatif tinggi yang terjadi di semua penelitian. Kesimpulan yang dapat diambil adalah penerapan *T. flavus* pada sisa-sisa bahan organik segar yang mengandung mikrosklerotium mampu mengurangi kepadatan inokulum *V. dahliae*. Perlunya penelitian lanjutan tentang pengaruh kepadatan populasi *T. flavus* terhadap laju perkembangan gejala penyakit layu yang disebabkan oleh *V. dahliae*.

Pada kaitannya dengan *T. flavus*, pengaruh strain bakteri *Pseudomonas fluorescens*, yang menghasilkan antibiotika 2,4-diacetylphloroglucinol, diuji pada Bab 6. *P. fluorescens* strain 60 (P60) menghambat paling sedikit 50% pertumbuhan miselium dari 20 isolat *V. dahliae* secara *in vitro* dan juga mengurangi pembentukan mikrosklerotium yang terjadi di sebagian besar isolat yang diuji. Pada uji hayati, *V. dahliae* secara nyata mempercepat penuaan *A. thaliana* dan terung. Akan tetapi, ketika tanaman tersebut diperlakukan dengan P60, penuaan melaju pada laju yang sama seperti pada perlakuan tanpa *V. dahliae*. Walaupun perlakuan tanaman-tanaman dengan P60 tidak berakibat utama pada pengurangan persentase bagian atas *A. thaliana* yang terinfeksi, jumlah mikrosklerotium yang dihasilkan oleh *V. dahliae* pada bagian atas tanaman yang tua berkurang secara nyata (sampai hampir 8 kali) di keempat penelitian. *P. fluorescens* tidak dapat diisolasi dari bagian dalam jaringan inang, tetapi *P. fluorescens* terdapat di daerah perakaran *A. thaliana* dan terung sampai 12 minggu setelah penginokulasian pada kepadatan yang beragam antara $1,4 \times 10^3$ - $8,0 \times 10^5$ upk per gram akar. Penggabungan P60 dengan *T. flavus* lebih jauh meningkatkan pengendali terhadap *V. dahliae*. Jika dibandingkan dengan perlakuan pembanding, jumlah mikrosklerotium yang terbentuk berkurang sampai hampir 40 kali lipat dengan adanya penerapan gabungan P60 dan *T. flavus*, dan pengendalian yang diperoleh dengan penggabungan tersebut secara nyata lebih baik daripada hasil pengurangan yang didapat dengan penerapan agensia tersebut secara terpisah.

Tiga pokok persoalan utama dikenal dalam diskusi umum (Bab 7): (1) penggunaan penerapan uji hayati untuk menghitung potensi inokulum *V. dahliae*, (2) faktor-faktor yang mempengaruhi jumlah mikrosklerotium di dalam tanah, dan (3) pengendalian *V. dahliae* secara hayati. Uji hayati pada dasarnya menyediakan informasi yang berbeda dari teknik teknik pencawanan yang hanya menghitung mikrosklerotium. Uji hayati yang diperlihatkan menampilkan kepekaan yang tinggi dan nampaknya sesuai untuk mendeteksi infestasi tanah yang rendah yang sukar dideteksi dengan metode yang lain. Selain itu, uji hayati sangat sesuai untuk digunakan pada kajian-kajian perbandingan penelitian. Faktor-faktor yang mempengaruhi jumlah mikrosklerotium di dalam tanah dapat dibedakan ke dalam faktor-faktor yang mempengaruhi pembentukan mikrosklerotium baru, dan yang mempengaruhi daya tahan hidup dan pemulihan jamur tersebut. Pada penentuan kepadatan mikrosklerotium di dalam tanah, pemulihan kemungkinan memainkan peranan penting, yang meskipun mengacaukan. Beberapa saran diusulkan untuk dilakukannya kajian lebih lanjut pada bidang ini, dengan penitik-beratan secara khusus pada pertanyaan berapa rendahkah laju pemulihan kemungkinan mempengaruhi proses-proses yang terkait di dalam tanah. Kesempatan yang jelas untuk mengembangkan metode pengendalian layu vertisilium dengan menggunakan antagonis telah tersedia. Strain-strain yang berbeda dari kedua antagonis *P. fluorescens* dan *T. flavus* terhadap layu vertisilium dianjurkan untuk dilakukan penyaringan. Hal ini diduga bahwa pengurangan pembentukan mikrosklerotium baru di dalam jaringan tanaman bagian atas, yang dirangsang oleh kedua antagonis tersebut, adalah disebabkan oleh suatu pengurangan jumlah infeksi akar oleh *V. dahliae*, atau melalui ketahanan yang dirangsang.

Author's curriculum vitae

The author was born on 26 of June 1960 at Pati, in the Province of Central Java, Indonesia. He received his junior and primary school at Sokaraja, Banyumas district, and moved to Purwokerto to obtain his secondary school at SMA Negeri I. In 1979 he enrolled at the University of Jenderal Soedirman, Purwokerto, from where he received his degree from the Faculty of Agriculture, the Department of Plant Pests and Diseases in January 1985. In March 1985 he started his career as a teacher at his almanater, the Department of Plant Pests and Diseases, the Faculty of Agriculture, the University of Jenderal Soedirman, Purwokerto. A Master degree course was taken at the University of Gadjah Mada, Yogyakarta, in 1986 and completed with a MSc of Phytopathology in April 1989. A PhD research project started in September 1995 at the Department of Phytopathology, Wageningen University, the Netherlands, with a scholarship from the Ministry of Education and Culture, the Republic of Indonesia, through the Higher Education Development Project (HEP II) - ADB Loan No. 1235-INO. After finishing the PhD research, he will continue his work as a teacher and researcher at the University of Jenderal Soedirman, Purwokerto.

Loekas Soesanto

Present address:

Department of Plant Pests and Diseases,
Faculty of Agriculture,
University of Jenderal Soedirman
P.O. Box 25
Purwokerto 53101
Indonesia

phone: +62 (0)281 38791